PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Schneck and Oelke	Group Art Unit: 1644
Serial No.: 10/618,267	Examiner: M. DIBRINO
Filed: July 14, 2003	Docket No.: 001107.00355
	Confirmation No. 3951

For: **REAGENTS AND METHODS FOR ENGAGING UNIQUE CLONOTYPIC LYMPHOCYTE RECEPTORS**

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. JONATHAN SCHNECK AND DR. MATHIAS OELKE

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

We, Jonathan Schneck and Mathias Oelke, declare as follows:

1. We are named as the inventors of the subject matter claimed in Serial No. 10/618,267. We are both employed by the Johns Hopkins University School of Medicine. Our professional biographies are attached as Exhibit 1 (Dr. Jonathan Schneck, Professor of Pathology and Medicine) and Exhibit 2 (Dr. Mathias Oelke, Assistant Professor of Pathology).

2. We understand that the U.S. Patent and Trademark Office has rejected claims of our patent application Serial No. 10/618,267 as obvious over several combinations of references,

including Latouche & Sadelain, "Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells," *Nature Biotechnology 18*, 405-09, 2000 ("Latouche"; Exhibit 3).

3. Latouche demonstrates that cell-based artificial antigen presenting cells (aAPC) can be made by transfecting NIH 3T3 cells with a tumor antigen and with relevant HLA A2 complexes. The cell-based aAPCs selectively stimulate growth of antigen-specific $CD8^+$ T cells. Latouche explains that there are a variety of co-stimulatory complexes, including the interaction of CD28 with B7-1 or B7-2, which are involved in stimulating the proliferation of antigen-specific $CD8^+$ cells and that the addition of cytokine plays an important role. Latouche also explains that the level of stimulation is based on HLA antigen density.

4. Latouche speculates that beads coated with anti-CD3 antibodies and with anti-CD28 antibodies ("anti-CD3/CD28 beads") might be used to expand populations of antigen-specific $CD8^+$ T cells: "If additional cells were needed or if the starting cell number was less, a third round of stimulation or further nonspecific activation using, for example, beads coated with anti-CD3and anti-CD28 antibodies could be envisaged."[1]

5. In contrast to the speculation in Latouche, anti-CD3/CD28 beads in fact do not support long-term growth of purified $CD8^+$ T cells. The failure of bead-based artificial antigen presenting "cells" to support proliferation of $CD8^+$ T cells is directly addressed in several papers, each of which was published before July 12, 2002. The experiments reported in these papers demonstrate that beads or plates coated with anti-CD3/anti-CD28 antibodies do not support the long term growth or expansion of purified $CD8^+$ T cells.

[1] Latouche at page 408, paragraph bridging columns 1 and 2, internal reference omitted. That reference (reference 30; Levine *et al.*, "Effects of CD28 costimulation on long-term proliferation of $CD4^+$ T cells in the absence of exogenous feeder cells," *J. Immunol. 159*, 5921-30, 1997; Exhibit 4) demonstrates that anti-CD3 beads support expansion of $CD4^+$ T cells.

6. Deeths[2] (Exhibit 5) investigated the effect on populations of $CD4^+$ and $CD8^+$ T cells of purified B7-1 (the ligand for the CD28 receptor) and anti-T cell receptor monoclonal antibodies immobilized on latex microspheres. Deeths found a dramatic difference between the responses of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ responses were sustained, and proliferation and expansion continued through day 6. Paragraph bridging pages 602 and 603 and Figures 3A, 3C, 3E. In contrast, although significant clonal expansion of $CD8^+$ T cells occurred after 4 days of stimulation with the microspheres, "after day 4, the expanded cells died rapidly, such that by day 6, the number of viable cells in the culture was nearly equal to the number present at the initiation of culture." Page 602, col. 2 ¶ 1 and Figures 3B, 3D, and 3F.

7. Laux[3] (Exhibit 6) demonstrated that $CD4^+$ T cells respond to anti-CD3/CD28 beads "with sustained proliferation," whereas $CD8^+$ T cells "grew for a limited period only." Abstract. $CD8^+$ T cells "had a markedly reduced growth potential in this culture system and became nonresponsive after 2 to 3 weeks of culture, as indicated by a plateau on the growth curve (Fig. 1A)." Page 189, col. 2. Laux discusses the fact $CD4^+$ cells can be expanded *ex vivo* using the anti-CD3/CD28 beads and used to treat HIV-1-infected patient but, "[s]hould a similar approach be desired for the $CD8^+$ subset . . . this protocol will need to be modified because of the poor proliferative response of the $CD8^+$ T-cells to CD28 costimulation." Page 193, col. 1.

8. The failure of anti-CD3/CD28 beads to support $CD8^+$ T cell proliferation cannot be overcome by the addition of interleukin 2 (IL-2) to the culture medium, as shown in Figure

[2] Deeths & Mescher, "B7-1-dependent co-stimulation results in qualitatively and quantitatively different responses by $CD4^+$ and $CD8^+$ T cells," *Eur. J. Immunol.* 27, 598-608, 1997.

[3] Laux *et al.*, "Response Differences between Human $CD4^+$ and $CD8^+$ T-Cells during CD28 Costimulation: Implications for Immune Cell-Based Therapies and Studies Related to the Expansion of Double-Positive T-Cells during Aging," *Clin. Immunol.* 96, 189-97, 2000.

2A of Maus[4] (Exhibit 7). Figure 2A demonstrates that anti-CD3/CD28 beads only support a single round of $CD8^+$ T cells. After that point, the beads no longer support CD8 cell expansion. These findings led the authors of Maus to develop a cell-based non-antigen specific aAPC (K562 cells which were genetically modified to express the low affinity Fc-Receptor through which anti-CD3mAb and anti-CD28mAb were bound to the surface, as well as the co-stimulatory molecule 4-1BB ligand; closed circles in Figure 2A). These genetically modified non-specific antigen-presenting cells were able to cause large scale expansion of $CD8^+$ T cells but still not large scale expansion of antigen specific T cells. The major difference between K32/4-1BBL cell-based aAPCs and the anti-CD3/CD28 beads is the membrane fluidity of the cells.

9. The evidence that anti-CD3/antiCD28 beads do not support repeated and sustained stimulation of $CD8^+$ T cells directly contradicts the speculation of Latouche. Based on the findings of the Laux, Deeths, and Maus, one would have predicted that a bead-based aAPC would not effectively to support $CD8^+$ expansion because it appeared that $CD8^+$ T cell expansion requires the interaction of $CD8^+$ T cells with other fluid membranes, as used by Latouche and by Maus.

10. Another unexpected limitation of non specific expansion of antigen specific $CD8^+$ T cells using anti-CD3CD28 beads, is the loss of antigenic-specificity. For example, during their attempt to expand antigen-specific $CD8^+$ T cells, Maus noted a significant drop in specific frequency of antigen-specific CTL cells (Exhibit 7, page 145, paragraph bridging columns 1 and 2). Maus reported that after tetramer sorting, which typically yields 95% or greater antigen

[4] Maus *et al.*, "*Ex vivo* expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB," *Nature Biotechnology 20*, 143-48, 2002.

specific $CD8^+$ T cells, the frequency of those antigen-specific T cells dropped to ~60% on day 17 then to ~20% over the course of expansion. This result was observed with multiple donors.

11. We also found a similar phenomenon when we compared anti-CD3/CD28 bead-based $CD8^+$ T cell expansion with the $CD8^+$ T cell expansion obtained using beads described in claim 1 of our application Serial No. 10/618,267. This experiment is described in Example 4 and outlined in Figure 1 of our application Serial No. 10/618,267. Example 4 refers to these beads as "aAPC"; as explained in Example 1 (paragraph 160) the "aAPC" used in this experiment are microbeads to which anti-CD28 antibody and "HLA-Ig (as described in U.S. Patent 6,268,411)" are coupled. "HLA-Ig" is the MHC class I-immunoglobulin complex recited in claim 1 of our application Serial No. 10/618,267. Thus, this experiment provides a direct comparison between the anti-CD3/CD28 beads postulated in Latouche and the beads claimed in this application.

12. In this experiment we found that the number of antigen-specific $CD8^+$ T cells dropped from approximately 88% antigen-specific CTL to 7.26%, less than 10% antigen-specific CTL following a single round of stimulation with anti-CD3/CD28 beads. See Figures 4A and 4B in the application. Results of a similar experiment are shown in Exhibit 8; compare panel A, bottom, to panel B, bottom.

13. In contrast, when we repeated the experiment with the HLA A2-based aAPC we found both proliferation and maintenance of the antigen-specific $CD8^+$ CTL cultures. See Figure 4C of the present application and compare panel A bottom to panel C, bottom of Exhibit 8.

14. In view of the work of Deeths, Laux, and Maus discussed above, we were quite surprised when our HLA-Ig based aAPC beads did support large scale antigen-specific $CD8^+$

expansion. These beads do not function similar to anti-CD3/CD28 beads nor could their properties have been predicted from data obtained using the beads.

15. Based on our studies with MHC class I-Ig beads and expansion of $CD8^+$ cells, we expect that beads containing MHC class II-immunoglobulin complexes will effectively expand antigen-specific $CD4^+$ cells.

16. All statements we made in this declaration of our own knowledge are true. We believe all statements made on information and belief to be true. We made these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent.

Dated: 04/02/08

Jonathan Schneck, M.D., Ph.D.

Dated: 04-02-08

Mathias Oelke, Ph.D.

Appendix V; revised 3/12/07

CURRICULUM VITAE FOR ACADEMIC PROMOTION

The Johns Hopkins University School of Medicine

(Signature) _____ _____
(Typed Name) Jonathan P. Schneck (Date of this version)

DEMOGRAPHIC AND PERSONAL INFORMATION:

Current Appointments:

2002 - present	Johns Hopkins University School of Medicine Professor of Oncology
May 2001 - present	Division of Immunopathology Department of Pathology Johns Hopkins University School of Medicine Professor of Pathology and Medicine

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Education and Training (in chronological order):

Year	Degree/Certificate	Institution	Discipline
1976	B.A.	Yeshiva University, New York, NY	
1976 – 1983	Med. Sci. Trainee	Albert Einstein College of Medicine Bronx, NY	
1983	M.D., Ph.D.	Albert Einstein College of Medicine & Sue Golding Graduate School of Biology, Bronx, NY	
1983 – 1986	Residency	Children's Hospital National Medical Center, Washington, D.C.	
1986 – 1989	Med. Staff Fellow	Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland	

Professional Experience (in chronological order, earliest first):

Dates	Positions	Institutions
1989 - July 1995	Assistant Professor of Medicine	Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University School of Medicine
1993 – 1998	Associate Editor	Journal of Allergy and Clinical Immunology
1993 – present	Member of Lucille P. Markey Graduate Program in Molecular and Cellular Medicine	Johns Hopkins University School of Medicine Baltimore, Maryland
1995 – 1996	Chairman of Immunology Council	Johns Hopkins University School of Medicine Baltimore, Maryland
1994 – present	Member of the Graduate Program Immunology	Johns Hopkins University School of Medicine Baltimore, Maryland
July 1995 - 2001	Associate Professor of Pathology and Medicine	Division of Immunopathology, Johns Hopkins University School of Medicine Baltimore, Maryland
May 2001 - present	Professor of Pathology and Medicine	Division of Immunopathology, Johns Hopkins University School of Medicine Baltimore, Maryland
2002 - present	Professor of Oncology	Johns Hopkins University School of Medicine Baltimore, Maryland

Honors and Awards:

1972 Semi-finalist in NASA Skylab Project
1973 – 1976 New York Regents Scholarship
1973 – 1976 Dean's List
1976 Phi Lambda Epsilon
1976 Magna Cum Laude
1976 – 1983 Medical Scientist Training Grant
1989 – 1991 Mellon Clinician Scientist Award
"The Role of Class I MHC Molecules in Immune Responses"
1990 – 1995 Harry Weaver Neuroscience Scholar Award
"Inhibition of T Cell Responses by MHC-Derived Peptides: A Novel Approach to Treatment of Autoimmune Diseases"

1990 RJR Research Scholars Award Program Finalist
1990 – 1993 Arthritis Investigators Award
"The Role of Class I MHC Molecules in the Regulation of T Cell Activation"
2003 – 2005 Multiple Myeloma Research Foundation Scholar's Award "Development of artificial antigen presenting cells for adoptive immunotherapy of multiple myeloma"

RESEARCH ACTIVITIES:

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5. **Schneck, J.**, Rosen, O., Diamond, B., and Bloom, B. R. Modulation of Fc-receptor expression and Fc-mediated phagocytosis in variants of a macrophage-like cell line. *J. Immunol. 1981; 126:745-74.*

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7. **Schneck, J.**, Rager-Zisman, B., Rosen, O. M., and Bloom, B. R. Genetic analysis of the role of cyclic AMP in interferon mediated events. *Proc. Natl. Acad. Sci. U.S.A. 1982; 79:1879-1883.*

8. Bloom, B. R., **Schneck, J.**, Rager-Zisman, B., Quan, P. C., Neighbour, A., Minato, N., Reid, L., and Rosen, O. M. Interaction between interferon and cells of the immune system. *In: Chemistry and Biology of Interferons: Relationship to Therapeutics - UCLA Symposia on Molecular and Cellular Biology, Vol. XXV. 1982; (Merrigan, T.C., and Friedman, R.M., editors). Academic Press, New York. p. 198.*

9. **Schneck, J.,** Maloy, W. L., Coligan, J. E., and Margulies, D. H. Inhibition of an allospecific T cell hybridoma by soluble class I proteins and peptides: Estimation of the affinity of a T cell receptor for class I MHC molecules. *Cell. 1989; 55:47-53.*

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11. Munitz, T. I., **Schneck, J.**, Coligan, J. E., Maloy, W. L., Henrich, J. P., Sharrow, S. O., Margulies, D. H., and Singer, A. A peptide derived from the alpha helical region of class I MHC blocks CTL engagement of the class I MHC molecule. *Cold Spring Harbor Symp. Quant. Biol. 1989; 54:557-561.*

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43. Hamad, A. R. A., and **Schneck, J**. Antigen Induced T Cell Death Is Regulated by CD4 Expression. *Intern. Rev. Immunol. 2001; 20:535-546.*

44. Greten, T. F., and **Schneck, J. P.** Development and use of multimeric major histocompatibility complex molecules. *Clin. Diag. Lab. Immunol. 2002; 9(2):216-20.*

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47. Greten, T. F., Korangy, F., Neumann, G., Wedemeyer, H., Schlote, K., Heller, A., Scheffer, S., Pardoll, D. M., Garbe, A. I., **Schneck, J. P.**, Manns, M.P. Peptide-β2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes. *J. Immunol. Methods 2002; 271:125-135.*

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50. Oelke, M., and **Schneck, J. P.** HLA-Ig based artificial Antigen-presenting cells: Setting the terms of engagement. *Clinical Immunology 2004; 10:243-251.*

51. Krueger, C., **Schneck, J.P.,** and Oelke, M. Quality and quantity: new strategies to improve immunotherapy of cancer. *Trends in Molecular Medicine 2004; 10:205-208.*

52. Oelke, M., and **Schneck, J. P.** Immunotherapy with enhanced self immune cells. *Discovery Medicine 2004; 4:203-207.*

53. Sirianni, N., Ha, P. K., Oelke, M., Califano, J., Gooding, W., Westra, W., Whiteside, T.L., Koch, W. M., **Schneck, J. P.**, DeLeo, A., and Ferris, R. L. Effect of human papillomavirus-16 infection on CD8+ T-cell recognition of a wild-type sequence p53264-272 peptide in patients with squamous cell carcinoma of the head and neck. *Clin. Cancer Res. 2004; 10:6929-37.*

54. Oelke, M., Krueger, C., and **Schneck, J. P.** Technological advances in adoptive immunotherapy. *Drugs of Today 2005; 41:13-21.*

55. Karabekian, Z., Simon, D., Lytton, Silver, P. B., Sergeev, Y., **Schneck, J. P.**, and Caspi, R. R. Antigen/MHC class II/Ig dimmers for study of uveitogenic T cells: IRBP p161-180 presented by both IA and IE molecules. *Investigative Ophthalmology & Visual Scienc, 2005; 46:3769-3776.*

56. Oelke, M., Krueger, C., Giuntoli, R. L., II, **Schneck, J. P.** Artificial antigen-presenting cells: artificial solutions for real diseases. *Trends in Molecular Medicine 2005; 11:412-420.*

57. Mohamood, A. S., Trujillo, C. J., Zheng, D., Jie, C., Martinez, M. F., **Schneck, J. P.**, Hamad, A. R. A. Gld mutation of Fas ligand increases the frequency and up-regulates cell survival genes in CD25+CD4+ TR cells. *Int. Immunol. 2006 Jun 12; [Epub ahead of print].*

58. Fahmy TM, **Schneck J.P.**, Saltzman WM.A nanoscopic multivalent antigen-presenting carrier for sensitive detection and drug delivery to T cells. Nanomedicine.2007.

59. Nagaraj1 S , Gupta K, Pisarev3 V , Kinarsky L, Sherman S, Loveleen K, Herber D , **Schneck J.P.**, and Gabrilovich D . Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nature Medicine 13, 828 – 835 2007.

60. Mohamood AS,Guler ML., Xiao Z, Zheng D, Hess A, Wang Y, Yagita H, **Schneck J.P.**, Hamad AR. Protection from autoimmune diabetes and T-cell lymphoproliferation induced by Fas L mutation are differentially regulated and can be uncoupled pharmacologically.Am J Pathol.2007.

61. Deviren G.,Gupta K.,Paulatis,M.E.,**Schneck J.P.** Detection of Antigen –Specific T cells on p/MHC Microarrays. J. Molecular Recognition 2007.

62. Schutz C, Fleck M, Mackensen A, Zoso A, Halbritter D, **Schneck J.P.**, Oelke M. Killer-artificial- antigen- presenting-cells (KaAPC): a novel strategy to delete specific T cells. Blood Journal 2007.

Inventions, Patents, Copyrights (pending, awarded):

Date	Title
01/18/00	DM 3085 - Soluble Divalent and Multivalent Heterodimeric Analogs of Proteins, and Molecular Complexes Which Modify Immune Responses. **Serial Number**: 08/828,712 **Patent Number:** 6,015,884
06/08/00	DM 3085 - Soluble Divalent and Multivalent Heterodimeric Analogs of Proteins, and Molecular Complexes Which Modify Immune Responses. **Serial Number**: 331688 **Patent Number**: 331688
10/31/00	DM-3085 - Polynucleotides encoding molecular complexes Which modify immune responses **Serial Number**: 09/063,276 **Patent Number:** 6,140,113
05/17/01	DM 3085 - Soluble Divalent and Multivalent Heterodimeric Analogs of Proteins, and Molecular Complexes Which Modify Immune Responses. **Serial Number**: 24224/97 **Patent Number**: 729406
09/10/02	DM 3085 - Soluble Divalent and Multivalent Heterodimeric Analogs of Proteins, and Molecular Complexes Which Modify Immune Responses. **Serial Number**: 09/324,782 **Patent Number**: 6,448,071
10/01/02	DM 3085 - Soluble Divalent and Multivalent Heterodimeric Analogs of Proteins, and Molecular Complexes Which Modify Immune Responses. **Serial Number**: 09/668,143 **Patent Number**: 6,458,354
05/11/04	DM 3302 - Use of Multivalent Chimeric Peptide-Loaded, MHC/IG Molecules to Detect, Activate or Suppress Antigen-Specific T Cell-Dependent Immune Responses. **Serial Number**: 09/789,720 **Patent Number:** 6,734,013

24 other patents are pending.

Extramural Funding (current, pending, previous)

CURRENT
01/01/91 - 03/31/08, Immunoregulatory functions of TCR and MHC complexes
RO1 AI29575
National Institutes of Health (Competitive Renewal)
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 33%
Notes:

05/01/03 – 11/30/06, Development of artificial antigen presenting cells for prostate cancer immunotherapy,
DAMD17-03-1-0370
DOD
Jonathan P. Schneck, M.D., Ph.D.
$
Principal Investigator, 15%
Notes:

09/30/03 – 08/31/08, SPORE in cervical cancer – Project 4,
5 P01 CA098252
National Institutes of Health
$
Tzyy Choou Wu, M.D., Ph.D.
Co-Investigator, 5%
Notes:

07/01/04 – 06/30/09,
Use of aAPC for melanoma adoptive immunotherapy
1 R01 CA108835
National Institutes of Health
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 20%
Notes:

09/10/04-07/31/06, B220+ DN □□ T cell as a novel immunoregulatory T cell
1R21 DK069279
National Institutes of Health
$
Abdel R. Hamad, DVM, Ph.D.
Co-Investigator, 4%
Notes:

4/01/05 – 12/31/09, Antigen specific modulation of T cell responses,
2 R01 AI44129
National Institutes of Health
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 20%
Notes:

PENDING
09/01/05 – 08/31/08, High throughout analysis of diverse T cell populations

National Institutes of Health
$
Michael Paulaitis, Ph.D.
Co-Investigator, 8%
Notes:

12/01/05 – 11/30/10, Inactivating Fas Pathway to prevent T cell autoimmunity
National Institutes of Health
$
Abdel R. Hamad, DVM, Ph.D.
Co-Investigator, 5%
Notes:

12/01/06-11/30/11, Antigen receptor inputs: linking structural, molecular and cellular responses
National Institutes of Health
$6,464,166
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 25%
Notes:

PREVIOUS
05/01/97 – 04/31/02, Mechanisms of accelerated graft arteriosclerosis (Pathology Core
1PO1 HL56091
National Institutes of Health
$
William M. Baldwin, III, M.D., Ph.D.
Co-Investigator, %

11/24/98 - 11/23/01, Research in the Area of In Vitro Diagnostic Applications
Pharmingen/Becton Dickinson (Funding Agreement)
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, %
Notes:

01/01/99 – 06/30/01, Activation of antigen-specific T cells as immunotherapy for the treatment of cancer and infectious diseases
Eligix, Inc.
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, %
Notes:

07/01/99 - 06/30/02, Factors controlling the T cell response to HIV Infection
Clinical Research Institute of Montreal (NIH)

$
Rafick Sekaly
Co-Investigator, %
Notes:

07/01/01 – 06/30/02,Development of an artificial antigen presenting cell
BioTransplant, Inc. (NIH)
%
Barbara Walner,
Co-Investogator, %
Notes:

01/01/01 - 12/31/02, Regulation of Bacteria-Induced Self-Reactive CD8 T cells
Arthritis Foundation (MARC)
Mark J. Soloski, Ph.D.
Co-Investigator, 3.5%
Notes:

04/01/99 – 05/31/04, Inhibition of antigen-specific T cell responses
RO1 AI44129
National Institutes of Health
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 18%
Notes:

10/01/03-09/30/04, Development of artificial antigen presenting cells for adoptive immunotherapy of Multiple Myeloma,
Multiple Myeloma Research Foundation
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 5%
Notes:

08/01/96 – 07/31/05, Strain specific CTL response in acute hepatitis C
U19 AI40035
Subcontract with University of Texas (NIH)
$
Thomas
Co-Investigator, 2%
Notes:

09/30/02 - 08/31/05, Generation of Artificial Antigen Presenting Cells
1 R21 HL72150-01
National Institutes of Health

$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 10%
Notes:

01/21/04 – 12/31/05, B220-DN Tcells in Mucosal Tolerance and Inflammation
1 R21 DK066039
National Institutes of Health
$
Abdel R. Hamad, DVM, Ph.D.
Co-Investigator, 2%
Notes:

04/01/04-01/31/06, Immune function in desensitized allograft recipients
1 R21 AI59920
National Institutes of Health
$
Jonathan P. Schneck, M.D., Ph.D.
Principal Investigator, 6%
Notes:

Research Program Building / Leadership:
Dates, name of research / basic science program, role

EDUCATIONAL ACTIVITIES:

Teaching:

1. Course Director First Year Medical Student Immunology Course. Principles of Immunology, Course #250.603.

2. Graduate School Immunology Course. Graduate Immunology, Course #250.703.

3. Advanced Topics in Immunology, Course #250.702.

4. Second Year Medical School Course. Physical Diagnosis.

5. Frontiers in Research and Clinical Management of Asthma and Allergy "from bench to bedside". Johns Hopkins Asthma and Allergy Center.

Mentoring (pre- and post-doctoral):

Advisees

Name	Dates	Degree	Present Position
Peebles, Ray Stokes	6/91 - 7/92	M.D.	Assistant Professor Dept. of Medicine

Johnson, Telt	6/91 - 6/93	Ph.D.	Vanderbilt University Head, Molecular Pharmacology Neuroscience, Denmark.
Catipovic, Branimir	10/90 - 6/94	M.D.	Clinical Fellow, Clincial Immunology (SOM)
Su, Xiao-Min	6/93 - 7/96	Ph.D.	
O'Herrin, Sean M.	8/94 - 12/98	Ph.D.	Instructor, University of Wisconsin
Vuica, Milena	8/94 - 5/97	M.D.	Pathology Resident, JHMI
Lebowitz, Michael	12/95 - 10/98	Ph.D.	Senior Scientist, IGEN
Greten, Tim	9/96 - 10/98	M.D.	Oncology Resident in Hannover, Germany
Slansky, Jill	9/96 - 01/02	Ph.D.	Postdoctoral Fellow, Department of Pathology, JHMI
Hamad, Abdel	5/97 - 1/00	Ph.D.	Research Associate, Department of Pathology, JHMI
Armstrong, Todd	10/97 - 09/01	Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI
Fahmy, Tarek	3/98 - present	M.S.	Predoctoral Student, IPMB, JHU
Menssen, Antje	6/98 - present	Ph.D.	Postdoctoral Fellow, Dept. of Oncology, JHMI
Peng, Shiwen	11/99 - present	M.D., Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI
Huhn, Karen	12/99 - 12/01	M.D.	Assistant Professor, Dept. of Dermatology and Pathology
Oelke, Mathias	11/00 - 7/03	Ph.D.	Instructor, Dept. of Pathology, JHMI
Russwurm, Georg	2/01 - 12/03	M.D., Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI (Dr. Russwurm is fully funded by Deutsche Forschungsgemeinschaft [The Germain Research Council])
Zemon, Harry	2001 - 6/2003	M.D.	Surgery Resident, George Washington University

Krueger, Christine	7/03 - 12/03	Graduate Student, Hanover University Med. Ctr.
Mohamood, Abdiaziz	2002 - present Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI
Gupta, Kapil	2003 - present	Graduate Student, Dept. of Pathology, JHMI
Durai, Malarvizhi	2004 - present Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI
Zoso, Alessia	2004 - present Ph.D.	Postdoctoral Fellow, Dept. of Pathology, JHMI
Heckel, Diane	2004 - present	Visiting Grad. Student, Dept. of Pathology, JHMI

• Thesis committees (provide: dates, name, title, your role)

• Training grant participation (provide: dates, program)

Dates	Program Name
9/1/82 - 8/31/04	Cellular & Molecular Mechanisms of Immune Inflammatory Reactions
7/1/99 - 6/30/01	Basic & Clinical Immunology Training Grant
7/1/00 - 6/30/05	Training Grant for Ph.D. Cancer Research Training for Post-Genomic Era
11/23/98	Graduate Program in Pathobiology
1992 - present	Cellular & Molecular Medicine Markey Trust Fund
9/1/99 - 8/31/03	Predoctoral Emphasis Pathway in Tumor Immunology From Cancer Research Institute
07/01/01 - 06/30/06	Training Program in the Pathobiology of Cancer
09/01/03 - 08/31/07	Emphasis in Tumor Immunology
05/01/03 - 04/30/08	Multidisciplinary Rheumatology Training Program

CURRICULUM VITAE

Signature	
Name	Mathias Oelke Ph.D.
Date	07-24-07
Current Appointment	Assistant Professor in the Department of Pathology, Johns Hopkins University School of Medicine
Personal Information	
Birthdate:	Mai 01, 1967
Birthplace:	Hildesheim, Germany
Citizenship:	German
Marital Status:	married to Ulrike Schlichte-Oelke, nee Schlichte, 2 children
Business Address	Johns Hopkins University School of Medicine Department of Pathology Ross Research Bldg Room 644 B 720 Rutland Ave Baltimore, MD 21205-2196
Phone	410-502-7128
Fax	410-614-3548
E-mail	moelke1@jhmi.edu

Education and Appointments

Since Sep. 2004	Assistant Professor in the Department of Pathology, Johns Hopkins University School of Medicine
Nov.2003 -Sep. 2004	Instructor in the Department of Pathology, Johns Hopkins University School of Medicine
July-Nov 2003	Research Associate in the Department of Pathology, Johns Hopkins University School of Medicine
Nov. 2000-2003	Post.-Doct.-Fellow at the Department of Pathology, Johns Hopkins University School of Medicine
Mai-Nov. 2000	Post.-Doct.-Fellow at the Department of Gastroenterology/Hepatology, Freiburg University Medical Center
Oct. 1996 – 2000	Doctoral Training under the direction of Prof. Andreas Mackensen and Prof. Albrecht Lindemann, Deparment of Hematology /Oncology, Freiburg University Medical Center: *"Induction, expansion and enrichment of human peptide-specific CD8+ T lymphocytes for adoptive T cell transfer in malignant melanoma"*
Jan. 1995 – Oct. 1996	Diploma Training under Dr. Hans Brachwitz at the Max Delbrueck Center for Molecular Medicine, Berlin-Buch: "*Influence of new cytostatically active phosphoserine analogs in single steps of the inositol signal cascade in normal and tumor cells"*
1990 - 1996	Student of Chemistry at the Technical University of Berlin
1987 - 1990	Student of Chemistry at the Technical University of Clausthal, Germany
1986 - 1987	Military Service with the German Army

RESEARCH ACTIVITIES

Publications

Abstracts

1. A. Mackensen, S. Wittnebel, **M.Ölke**, H. Veelken, F. Rosenthal, R. Mertelsmann and A. Lindemann. Induction and large scale expansion of $CD8^+$ tumor-specific CTL by in vitro stimmulation with CD80-transfected autologous melanoma cells
   Abstract of the 28th Annual Meeting of the Deutsche Gesellschaft für Immunologie, Würzburg 1997. Immunobiology Volume 197, Number 2-4, 347, September 1997

2. **M.Ölke**, U. Möhrle, A. Lindemann and A. Mackensen
   Induction and expansion of peptide-specific human $CD8^+$ T cells in vitro for adoptive immunotherapy of cancer
   Abstract of the 29th Annual Meeting of the Deutsche Gesellschaft für Immunologie, Freiburg 1998. Immunobiology Volume 199, Number 3-5, 621, September 1998

3. T. Kurokawa, A. Mackensen, **M. Ölke**, M. Lübbert, R. Mertelsmann and A. Lindemann. Analysis of T cell receptor variability in peripheral blood lymphocytes from a human regressive renal cell carcinoma
   Abstract of the 29th Annual Meeting of the Deutsche Gesellschaft für Immunologie, Freiburg 1998. Immunobiology Volume 199, Number 3-5, 618, September 1998

4. **M.Ölke**, U. Möhrle, A. Lindemann and A. Mackensen
   Generation of peptide-specific human $CD8^+$ T cells in vitro for an adoptive Transfer in malignant and infectious diseases
   Abstract of the Meeting of the Deutsche und Östereichischen Gesellschaften für Hämatologie und Onkologie, Frankfurt 1998
   Annals of Hematology, Supplement II to Volume 77 (1998), 82

5. T. Kurokawa, A. Mackensen, **M. Ölke**, M. Lübbert, R. Mertelsmann and A. Lindemann. Clonal expansion of $CD8^+$ T cells in peripheral blood lymphocytes from a human regressive renal cell carcinoma Abstract of the Meeting of the Deutsche und Östereichischen Gesellschaften für Hämatologie und Onkologie, Frankfurt 1998. *Annals of Hematology, Supplement II to Volume 77 (1998), 82*

6. **M.Ölke**, U. Möhrle, D. Behringer, A. Lindemann and A. Mackensen. Generation and purification of $CD8^+$ Melan A-specific cytotoxic T lymphocytes for adoptive transfer in tumor immunotherapy
   Abstract of the 30th Annual Meeting of the Deutsche Gesellschaft für Immunologie, Hannover 1999. Immunobiology Volume 200, Number 3-5, 614, September 1999

7. Rösler KW, Schmieder W, Kurz G, Kist M, Schiltz E, **Ölke M**, Tuczek A, Dettenborn **T**, Kreisel W. Antibodies to β-subunit of bacterial RNA-polymerase in primary biliary cirrhosis.
Gastroenterology 2001; 120 Suppl 1; A-76.

8. **Ölke M**, Tuczek A, Behringer D, Männle H, Jensen D, Rösler KW, Kreisel W. Specific Th2-type reaction of T-lymphocytes of PBC patients to calreticulin and bacterial RNA-polymerase.
Gastroenterology 2001; 120 Suppl 1: A-355.

9. **Ölke M**, Tuczek A, Behringer D, Männle H, Jensen D, Rösler KW, Kreisel W. Th- 2-Reaktion peripherer T-Lymphozyten auf Calreticulin und bakterielle RNA-Polymerase bei Primär Biliärer Zirrhose.
Z Gastroenterol 2001; 39: 675.

10. Rösler KW, Schmieder W, Kurz G, Kist M, Batsford S, Schiltz E, **Ölke M**, Tuczek A, Dettenborn T, Behringer D, Kreisel W. Antikörper gegen die β-Untereinheit der bakteriellen RNA-Polymerase bei der Primär Biliären Zirrhose (PBC). Z Gastroenterol 2001; 39: 675.

11. Tuczek A, **Oelke M**, Jensen D, Maennle H, Roesler KW, Behringer D, Kreisel W. TH2-Type reaction of T-lymphocytes to highly conserved proteins: specific for PBC?
Gastroenterology 2002; 122 (Suppl.): A-445.

12. **Oelke M**, Maus MV, Didiano D, June C, Mackensen A, Schneck JP. Ex vivo expansion of antigen-specific cytotoxic T cells by HLA-Ig coated artificial Antigen Presenting Cells
Keystone Symposia 2003; Basic Aspects of Tumor Immunology (C5):

13. **Oelke M**, Krueger C, Durai M, Bennett JD, Mackensen A, Schneck JP. In vivo function of antigen-specific cytotoxic T cells induced and expanded with HLA-Ig coated artificial Antigen Presenting Cells
Keystone Symposia 2004; Immune Evasion (X6): 122

14. **Oelke M**, Krueger C, June C, Bennett JD, Mackensen A, Schneck JP. Generation and function of antigen-specific cytotoxic T cells induced by HLA-Ig coated artificial Antigen Presenting Cells
The FASEB Journal; Experimental Biology 2004; Part I: 85.28

15. Malar Durai, Nick, P. Restifo, Jonathan P. Schneck and **Mathias Oelke** Functional characteristics of antigen specific CTL from pmel-1 transgenic mice activated using artificial Antigen Presenting Cells (aAPC)
Cellular Therapy: Third International Symposium on the Clinical use of Cellular Products, Regensburg March 17-18, 2005

16. M. Durai, C. Krueger, Z. Ye, L. Cheng, A. Mackensen, **M. Oelke** and J.P. Schneck In vivo anti-tumor efficacy of melanoma antigen-specific cytotoxic T lymphocytes generated using artificial antigen presenting cells.
Annual meeting of the American Association of Immunologist May 2006 in Boston

17. A. Zoso, S. Peng, J. D. Bennett, D. Heckel, J. P. Schneck and **M. Oelke**
Dimeric ^{peptide}MHC-Ig complexes: a novel strategy to activate antigen-specific T cells in vivo.
Annual meeting of the American Association of Immunologist May 2006 in Boston

18. B. Gertz, Y. Jeong, A. Zoso, J. Schneck and **M. Oelke**
Development of high-throughput assay for detection of low frequency memory CD8 cells: possible uses in cancer immunotherapy and vaccine development.
Annual meeting of the American Association of Immunologist May 2006 in Boston

19. T. Webb, J.P. Schneck and **M. Oelke**
Ex vivo induction and expansion of Human Natural Killer T cells by CD1d1-Ig coated artificial antigen presenting cells.
Annual meeting of the American Association of Immunologist May 2006 in Boston

20. Alessia Zoso, Carmela De Santo, Ilaria Marigo, Jonathon Drew Bennet, Paola Zanovello, Jonathan P. Schneck, Vincenzo Bronte, and **Mathias Oelke**
aAPC immunization activates low affinity T cells for treatment of tumors
Annual meeting of the American Association of Immunologist May 2007 in Miami

21. Tonya J Webb, Ophelia Rogers, Robert L. Giuntoli, II, Jonathan Schneck, and **Mathias Oelke**
Tumor Associated Ascites Inhibits CD1d-Mediated Antigen Presentation
Annual meeting of the American Association of Immunologist May 2007 in Miami

Peer reviewed original publication

1. Brachwitz, H., **Olke, M.,** Bergmann, J. and Langen, P.: Alkylphospho L- serine analogues: synthesis of cytostatically active alkylphosphono derivatives. Bioorg.Med.Chem.Lett. 7: 1739-1742, 1997.

2. **Ölke M.,** U. Möhrle, D. Behringer, A. Lindemann, and A. Mackensen. Generation and purification of Melan-A-specific human CD8+ T cells in vitro for an adoptive transfer in tumor immunotherapy. Clin. Cancer Res., 6: 1997-2005, 2000.

3. **Oelke M.**, T. Kurokawa, I. Hentrich, D. Behringer, V. Cerundolo, A. Lindemann, and A. Mackensen. Functional characterization of CD8+ antigen-specific cytotoxic T lymphocytes after enrichment based on cytokine secretion: comparison with the MHC-tetramer technology. Scand. J. Immunol., 52: 532-537, 2000.

4. Kurokawa T., **M. Oelke,** and A. Mackensen. Induction and clonal expansion of tumor- specific cytotoxic T lymphocytes from renal cell carcinoma patients after stimulation with autologous dendritic cells loaded with tumor cells. Int. J. Cancer, 91:749-756, 2001.

5. Roesler KW, Schmieder W, Kurz G, Kist M, Batford S, Schiltz E, **Oelke M,** Tuczek A, Dettenborn T, Behringer D, Kreisel W. Identification of β-subunit of bacterial RNA-Polymerase-a non species-specific bacterial protein- as target of

antibodies in primary billiary cirrhosis. Digestive Diseases and Science 2003 Mar;48(3):561-9

6. **Mathias Oelke**, Marcela V. Maus, Dominic Didiano, Carl June, Andreas Mackensen and Jonathan P. Schneck. Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig coated artificial Antigen Presenting Cells. Nature Medicine 2003 May; 9(5):619-24

7. **Mathias Oelke**, Jonathan Schneck. HLA-Ig based artificial Antigen-presenting cells: Setting the terms of engagement. Journal of clinical immunology 2004; Vol 110/3 pp 243-251

8. **Mathias Oelke**, Christine Krueger and Jonathan P. Schneck Technological Advances in Adoptive Immunotherapy Drugs Today (Barc). 2005 Jan;41:13-21.

9. Christine Krueger, Jonathan P. Schneck and **Mathias Oelke** QUALITY AND QUANTITY: NEW STRATEGIES TO IMPROVE IMMUNOTHERAPY OF CANCER. Trends in Molecular Medicine 2004 May;10(5):205- 8.

10. Sirianni N, Ha PK, **Oelke M**, Califano J, Gooding W, Westra W, Whiteside TL, Koch WM, Schneck JP, DeLeo A, Ferris RL.
Effect of human papillomavirus-16 infection on CD8+ T-cell recognition of a wild-type sequence p53264-272 peptide in patients with squamous cell carcinoma of the head and neck. Clin Cancer Res. 2004 Oct 15;10(20):6929-37.

11. **Mathias Oelke**, Christine Krueger, Robert L. Giuntoli II and Jonathan P. Schneck Artificial antigen-presenting cells: artificial solutions for real diseases *Trends in Molecular Medicine 2005 Sep;11(9):412-20.*

12. Boin F, Wigley FM, Schneck JP, **Oelke M**, Rosen A.
Evaluation of Topoisomerase-1-Specific CD8+ T-Cell Response in Systemic Sclerosis. Ann N Y Acad Sci. 2005 Dec;1062:137-45

13. Choi KS, Webb T, **Oelke M**, Scorpio DG, Dumler JS.
Differential Innate Immune Cell Activation and Proinflammatory Response in Anaplasma phagocytophilum Infection.
Infect Immun. 2007 Jun;75(6):3124-30. Epub 2007 Apr 2.

14. Ananta Paine, **Mathias Oelke**, Rainer Blasczyk, Britta Eiz-Vesper
Expansion of human cytomegalovirus-specific T lymphocytes from unfractionated peripheral blood mononuclear cells using artificial antigen presenting cells.
Accepted for publication in Transfusion

Non-peer review publication

1. **Mathias Oelke,** Toshiro Kurokawa, Andreas Mackensen. Functional analysis of antigen-specific CTL after enrichment based on cytokine secretion. MACS&more Vol. 5 No. 1 2001

Patent
Reagents and Methods for Engaging unique clonotypic Lymphocyte Receptors (pending; submitted July 2003)

Extramural Funding (current, previous)

Current Funding

W81XWH0510133 PI: Oelke **11/19/04-11/18/07**
DOD $100.000

Title: Development of artificial antigen presenting cells for prostate cancer immunotherapy

Notes: The major goal of this grant is to generate PSMA prostate cancer-specific CTL *in vitro* using artificial Antigen Presenting Cells and to evaluate the *in vivo* efficacy of these aAPC induced prostate cancer-specific CTL after adoptive T cell transfer in a human/SCID model.

R01 AI44129 PI: Schneck **04/01/05 -12/31/09**

NIH/NIAID $170.000

Title: Antigen specific modulation of T cell responses

Role: Co-Investigator

Notes: The major goals of this project are to analyze the structural basis of degenerate TCR recognition; design new approach to identify and track antigenic peptide/MHC complexes; and construct additional soluble high affinity TCR and MHC analogs.

R01 CA108835 PI: Schneck **07/01/04 -06/30/09**

NIH $205,000

Title: Use of aAPC for melanoma adoptive immunotherapy

Role: Co-Investigator,

Notes: The major goals are to develop aAPC that are optimized to expand T cells from patient with advanced melanoma.

Pilot grant in Cancer-related research Oelke (PI) 06/01/07 – 05/31/09

Kimmel Cancer Center at JHU (sponsored by the Stewart Trust Foundation) $50,000

Title: Development of novel strategies for adoptive immunotherapy of breast cancer

Notes: The goal of the grant is the development of new strategies for active and adoptive immunotherapy in Breast cancer.

Previous Funding

Brian D. Novis Award PI: Oelke 01/01/04 – 12/31/05

IMF $40,000

Title: Development of artificial antigen presenting cells for adoptive immunotherapy of multiple myeloma

Notes: The Major goals of this project are we propose to study use of HLA-Ig based aAPC for induction and expansion of multiple myeloma specific CTL for adoptive immunotherapy in multiple myeloma.

MRI-Pilot grant PI: Oelke 01/03/2005 – 29/02/2007

Johns Hopkins Malaria Research Institute $99,838

Title: Novel approach for detection of low frequency Malaria-specific T cells

Notes: The major goal of this grant is to develop a new approach to detect antigen specific T cells at very low frequencies.

DAMD17-03-1-0370 PI: Schneck 05/01/03 -11/30/06

DOD $125,000

Title: Development of artificial antigen presenting cells for prostate cancer immunotherapy

Role: Co-Investigator,

Notes: Development of artificial antigen presenting cells for adoptive immunotherapy for prostate cancer

EDUCATIONAL ACTIVITIES

Invited Review article (per review)

Mathias Oelke, Christine Krueger and Jonathan P. Schneck Technological Advances in Adoptive Immunotherapy, Drugs of Today, 41:13-21, Jan. 2005.

Mathias Oelke, and Jonathan P. Schneck.
Immunotherapy With Enhanced Self Immune Cells,Discovery Medicine, 4 (22): 203-7, June-August 2004.

Mathias Oelke, Christine Krueger, Robert L.Giuntoli II and Jonathan P. Schneck
Artificial antigen-presenting cells (aAPCs): artificial solutions for real diseases, TMM (accepted for publication in July 2005).

Organizational Activities

Editorial Activities

Reviewer for the Journal of Immunotherapy (2005, 2006 and 2007)

Reviewer for the Journal of Nanoscience and Nanotechnology (2006)

Reviewer for the NIH/NIAID for "Innovative Grants on Immune Tolerance" (Jan. 2006)

Memberships

Member of the American Association of Immunologist since 2004

Member of the German Association of Hematology and Immunology (DGHI) since 2007

Member of the American Association for the Advancement of Science (AAAS) since 2007

RECOGNITION

Awards

Winner of the "Fourth Annual Pathology Young Investigators' Day Award" for Excellence in Basic Research

Two year Post. Doct. Training Grant from the "Dr. Mildred-Scheel-Stiftung Deutsche Krebshilfe" (German Cancer Foundation)

Winner of the " 2004 Brian D. Novis Research Award – Junior grant"

Invited Review article

Mathias Oelke, Christine Krueger and Jonathan P. Schneck Technological Advances in Adoptive Immunotherapy, Drugs of Today, 41:13-21, Jan. 2005.

Mathias Oelke, and Jonathan P. Schneck.
Immunotherapy With Enhanced Self Immune Cells,Discovery Medicine, 4 (22): 203-7, June-August 2004.

Mathias Oelke, Christine Krueger, Robert L.Giuntoli II and Jonathan P. Schneck
Artificial antigen-presenting cells (aAPCs): artificial solutions for real diseases, TMM (accepted for publication in July 2005).

Invited Talks

Induction and Expansion of Peptide-Specific Human $CD8^+$ T cells in vitro for Adoptive Immunotherapy of Cancer
5th EUCC SYMPOSIUM
European Cancer Center (Basel-Freiburg-Strasbourg)

Generation and purification of $CD8^+$ Melan A-specific cytotoxic T lymphocytes for adoptive transfer in tumor immunotherapy
30th Annual Meeting of the Deutsche Gesellschaft für Immunologie, Hannover 1999

Ex vivo induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig coated artificial Antigen Presenting Cells
Cellular Therapy: Second International Symposium on the Clinical use of Cellular Products, Regensburg March 27-28, 2003

Antigen-specific T cells in Ovarian Cancer
Oophest meeting (monthly meeting of the Ovarian Cancer research Group at JHU)
Baltimore June 10th 2005.

The ABC of aAPC, artificial Antigen Presenting Cells: A new approach to adoptive immunotherapy
Pathology Grand Rounds at JHU
Baltimore July 18th 2005

aAPC immunization activates low affinity T cells for treatment of tumors
4th International Symposium on the clinical use of Cellular Products – Cellular Therapy 2007
Regensburg, Germany March 22nd 2007

Development of a new approach for detection of low frequency Malaria-specific T cells
Malaria Interest Group Meeting
Baltimore Mai 18th 2007

Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells

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The adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTLs) is a promising therapeutic approach for a number of diseases. To overcome the difficulty in generating specific CTLs, we established stable artificial antigen-presenting cells (AAPCs) that can be used to stimulate T cells of any patient of a given human leukocyte antigen (HLA) type. Mouse fibroblasts were retrovirally transduced with a single HLA-peptide complex along with the human accessory molecules B7.1, ICAM-1, and LFA-3. These AAPCs consistently elicit strong stimulation and expansion of HLA-restricted CTLs. Owing to the high efficiency of retrovirus-mediated gene transfer, stable AAPCs can be readily engineered for any HLA molecule and any specific peptide.

Keywords: adoptive cell therapy, CD8+ T cell, costimulation, dendritic cells, immunotherapy, retrovirus-mediated gene transfer.

The infusion of antigen-specific T lymphocytes is a potential therapy against certain cancers and infectious diseases[1–3]. One limitation to its broad usage is the generation of autologous T cells directed against well-defined epitopes. The induction and expansion of antigen-specific T cells require optimal antigen presentation and T-cell costimulation[4,5]. These requirements are met by antigen-presenting cells (APCs) such as Epstein–Barr virus-transformed B cells and dendritic cells (DCs), which constitutively express high levels of costimulatory, adhesion, and major histocompatibility complex (MHC) molecules[4,5]. Despite a cumbersome generation process, the use of autologous cells to present well-defined epitopes is mandated to obviate strong allogeneic responses. Therefore, we have undertaken to generate artificial APCs (AAPCs) with a single MHC restriction that could be used for any patient sharing the same MHC molecule. Several distinct signals contribute to effectively initiate and sustain T-cell activation and proliferation. The T-cell receptor must engage the MHC–peptide complex, which provides the basis for antigen specificity[6]. Signaling through the CD28 receptor provides a powerful costimulatory signal following engagement of the B7.1 (CD80) or B7.2 (CD86) ligand[7]. The adhesion molecule ICAM-1 (CD54) provides a synergistic signal through the LFA-1 (CD11/CD18) molecule expressed on T cells, whereas other molecules, in particular LFA-3 (CD58), ligand of the T-cell molecule CD2, can also mediate costimulatory as well as adhesion functions[8,9]. These accessory molecules are expressed at high levels on DCs, which are able to induce naive T lymphocytes[4], and a major role of B7.1, ICAM-1, and LFA-3 in costimulating cytotoxic T lymphocytes (CTLs) has been reported[10–12]. These three human costimulatory and adhesion molecules were retrovirally transduced in xenogeneic mouse fibroblasts with a single human leukocyte antigen (HLA) molecule. To efficiently present MHC–peptide complexes to CTLs, single MHC class I molecules were coexpressed with human β_2-microglobulin and a single genetically encoded peptide. Starting from peripheral blood T cells harvested from HLA A2.1+ donors, we demonstrate potent induction and expansion of CTLs against viral and self-peptides presented in the context of HLA A2.1. Three epitopes derived from influenza matrix[13,14], MART-1[15], and gp100[16] proteins were investigated. Cytotoxicity was highly specific and increased by restimulation with the AAPCs. Induction of CTLs was more efficient than that obtained with autologous blood-derived DCs. Cytotoxic activity induced by AAPCs encoding the MART-1 or gp100-derived peptide was elevated against HLA A2.1+ (but not A2.1−) melanoma cell lines that express these antigens. These findings establish that high-level cell surface expression of B7.1, ICAM-1, LFA-3, and single MHC class I–peptide complexes is sufficient to effectively induce strong antigen-specific CTL responses in human peripheral blood cells. AAPCs should be useful for the investigation of primary T-cell activation and the generation of antigen-specific T cells for adoptive cell therapies.

Results

Construction of AAPCs. To generate AAPCs restricted to the HLA class I A2.1 molecule ($AAPC^{A2}$), replication-incompetent retroviral vectors were used to sequentially transduce NIH/3T3 fibroblasts with five vectors encoding, respectively, human B7.1, ICAM-1, LFA-3, human β2-microglobulin, and HLA A2.1 (Fig. 1A). To maximize and sustain expression of a specific HLA–peptide complex, a dicistronic vector encoding an HLA-restricted epitope and puromycin-*N*-acetyltransferase was used (Fig. 1A). The expression of the peptide, targeted to the endoplasmic reticulum by the human CD8 leader, was maintained under selective pressure with puromycin. High-level and stable expression of the different transmembrane molecules was obtained (Fig. 1B). By flow cytometry analysis, the levels of expression of A2.1, B7.1, ICAM-1, and LFA-3 were comparable to those measured on mature A2.1+ dendritic cells (data not shown).

Artificial APCs efficiently stimulate flu-specific cytotoxic T-cell responses. Peripheral blood T lymphocytes harvested from HLA A2.1+ donors were stimulated either with primary autologous DCs pulsed with the flu peptide or $AAPC^{A2}$ genetically engineered to express the same peptide ($AAPC^{A2/F}$). Highly purified populations of T cells were prepared by positive selection (sheep red blood cells

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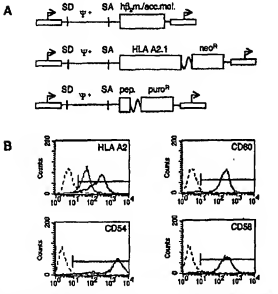

Figure 1. Generation of AAPCs from mouse fibroblasts. (A) Monocistronic retroviral vectors expressed human β2-microglobulin (hβ2m) and the accessory molecules (ecc.mol.) CD80, CD54, and CD58 (top). Dicistronic vectors were generated for HLA A2.1 and the peptide coding sequence (pep.), respectively linked by an internal ribosomal entry site to neomycin phosphotransferase (neo^R, middle) or puromycin-N-acetyltransferase (puro^R, bottom). SD, Splice donor site; SA, splice acceptor site; ψ+, extended packaging signal. (B) Flow cytometry analysis of HLA A2.1, CD80, CD54, and CD58 expression in AAPCs. The same cells are stained for each molecule as indicated. Solid lines correspond to transduced NIH 3T3 cells and dashed lines to untransduced cells. For HLA A2.1, the dotted line corresponds to cells transduced with HLA A2.1 without human β2-microglobulin, and the solid line to cells transduced with both cDNAs.

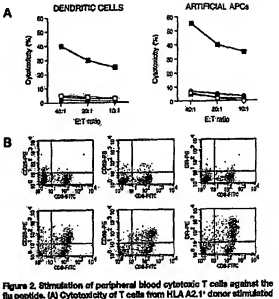

Figure 2. Stimulation of peripheral blood cytotoxic T cells against the flu peptide. (A) Cytotoxicity of T cells from HLA A2.1+ donor stimulated with primary autologous dendritic cells (left panel) or AAPC^Δ2 (right panel). Standard ^{51}Cr release assays were performed using TAP-deficient A2.1+ T2 target cells pulsed with the flu peptide (filled symbols) or the irrelevant MART-1 peptide (open symbols). Squares correspond to T cells stimulated against the flu peptide; circles to T cells stimulated without the relevant peptide. Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E/T) ratio. (B) Flow cytometry analysis of CD8+ T cells before (upper panels) and after (lower panels) cocultivation with HLA A2.1+ AAPCs encoding the flu peptide. T cells were stained with a fluorescein isothiocyanate (FITC)-labeled antibody against CD8 (x-axis) and, from left to right, phycoerythrin-labeled antibodies against CD28, CD69, and DR (y-axis). Results are from one of six experiments with one representative donor.

rosetting) and depletion of monocytes-macrophages, B cells, natural killer cells, and activated T cells (see Experimental protocol). After 8–10 days of stimulation, T lymphocytes cultured with AAPC^Δ2 exhibited strong flu-specific cytolytic activity (Fig. 2A). Typically the cytolytic activity was 1.6- to 4-fold higher than that obtained with primary dendritic cells pulsed with the flu peptide (115 and 65 lytic units, respectively, in Fig. 2A). The background on unpulsed target cells or on target cells pulsed with an irrelevant peptide was always lower than 5% (Fig. 2A). Examination of the cell surface phenotype of the CD8+ cells showed a strongly activated profile, as reflected by the high level of expression of CD25 (low-affinity interleukin-2 receptor), CD69 (very early activation marker), and HLA DR (Fig. 2B). Fewer than 5% of the purified T cells expressed these markers at the start of the coculture (Fig. 2B). Furthermore, absolute cell counts of CD8+ T cells on days 8–10 showed a higher cell yield following coculture with AAPCs than with primary DCs, about 2-fold higher in six different experiments ($P < 0.001$, Fig. 3). Such an expansion of CD8+ T cells could not be reached with AAPCs expressing ICAM-1 and/or LFA-3 in the absence of B7.1. On the other hand, the presence of both accessory molecules increased the effect of B7.1 by a factor of 2 (unpublished observations).

Artificial APCs efficiently induce CTLs specific for self-antigens. To address whether AAPCs could induce a response against self-antigens, HLA A2.1+ AAPCs encoding two peptides expressed in human HLA A2.1+ melanoma cells were generated. One peptide is derived from the MART-1 protein[19] and the other from the gp100 protein including an amino acid substitution to enhance binding to HLA A2.1[20]. Highly purified T cells harvested from three HLA A2.1+ donors were cultured with AAPCs expressing the MART-1 (AAPC^Δ2/1) or gp100 (AAPC^Δ2/2) derived peptide, using AAPC^Δ2 as control. After the first stimulation, as expected, a high response was obtained against the flu peptide in all three donors. In one donor, we readily detected a measurable CTL response against the MART-1 peptide (Fig. 4). After restimulation with the respective AAPCs, a readily detectable cytolytic response was obtained against all three peptides while the flu response further increased (Fig. 4). After restimulation, the response against the MART-1 peptide was of comparable magnitude to that obtained against the flu peptide after the first stimulation. The cytolysis obtained

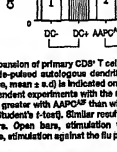

Figure 3. Expansion of primary CD8+ T cells stimulated with AAPC^Δ2 or flu peptide-pulsed autologous dendritic cells. CD8+ T-cell yield (fold increase, mean ± s.d) is indicated on the y-axis, corresponding to six independent experiments with the same donor. The yield was significantly greater with AAPC^Δ2 than with flu peptide-pulsed DCs ($P < 0.001$, Student's *t*-test). Similar results were obtained with two other donors. Open bars, stimulation without relevant peptide; hatched bars, stimulation against the flu peptide.

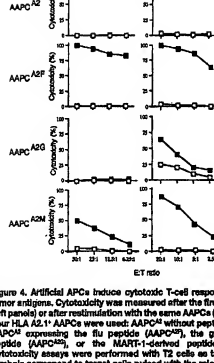

Figure 4. Artificial APCs induce cytotoxic T-cell responses against tumor antigens. Cytotoxicity was measured after the first stimulation (left panels) or after restimulation with the same AAPCs (right panels). Four HLA A2.1+ AAPCs were used: AAPC^{A2} without peptide (AAPC^{0}), AAPC^{A2} expressing the flu peptide (AAPC^{A2F}), the gp100-derived peptide (AAPC^{A2G}), or the MART-1-derived peptide (AAPC^{A2M}). Cytotoxicity assays were performed with T2 cells as targets. Filled symbols correspond to target cells pulsed with the relevant peptide; open symbols to target cells pulsed with an irrelevant peptide (MART-1 peptide for CTLs stimulated with AAPC^{A2}, flu peptide for CTLs stimulated with AAPC^{A2}, AAPC^{A2G} or AAPC^{A2M}). Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios.

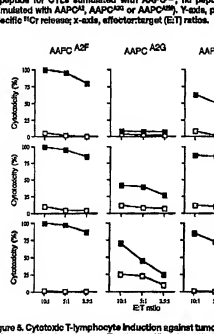

Figure 5. Cytotoxic T-lymphocyte induction against tumor antigens in different HLA A2.1+ donors. T cells purified from three HLA A2.1+ donors (A, B, C) were stimulated twice by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M}. Cytotoxicity stimulation was performed on T2 cells as described in Figures 2 and 4. y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios.

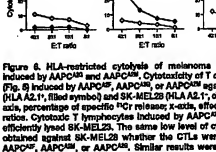

Figure 6. HLA-restricted cytolysis of melanoma cells by CTLs induced by AAPC^{A2G} and AAPC^{A2M}. Cytotoxicity of T cells of donor C (Fig. 5) induced by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M} against SK-MEL23 (HLA A2.1+, filled symbol) and SK-MEL28 (HLA A2.1-, open symbol). Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios. Cytotoxic T lymphocytes induced by AAPC^{A2G} and AAPC^{A2M} efficiently lysed SK-MEL28. The same low level of cytotoxicity was obtained against SK-MEL28 whether the CTLs were activated on AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M}. Similar results were obtained with donor B of Fig. 5 (data not shown).

after two stimulations for the three peptides in three HLA A2.1+ donors is shown in Figure 5. All three donors showed strong responses against the MART-1 peptide, and two out of three significantly responded to the gp100 peptide. Results obtained with these three donors in terms of cellular expansion and antigen specificity for all three peptides investigated in this study are summarized in Table 1. After two rounds of stimulation with AAPC^{A2F}, CD8+ T-cell yields increased 25- to 80-fold. After two rounds of stimulation with AAPC^{A2G} or AAPC^{A2M}, CD8+ T-cell yields increased 8- to 30-fold. CD8+ T cells were highly activated, as indicated by their elevated expression of CD25, CD69, and HLA DR (with phenotypic profiles similar to those shown in Figure 2B).

Cytotoxic T lymphocytes induced by AAPC^{A2} that encode the MART-1 or gp100-derived peptide specifically lyse HLA A2.1+ melanoma cells. To address whether T cells induced by AAPCs recognize and lyse melanoma cells in an HLA-restricted manner, cytotoxicity assays were performed using HLA A2.1+ and HLA A2.1- melanoma cells as targets. The SK-MEL23 and SK-MEL28 cell lines both express MART-1 and gp100 proteins and are, respectively, A2.1+ and A2.1- (*ref.* 21). T cells induced by AAPC^{A2G} or AAPC^{A2M} effectively lysed SK-MEL23 cells, showing, respectively, 30 and 45% lysis at the 40:1 effector:target ratio (Fig. 6). These T cells were HLA restricted as they failed to lyse SK-MEL28. On the other hand, T cells stimulated by AAPC^{A2F} failed to lyse SK-MEL23, demonstrating their high specificity. The low-level cytoxicity against SK-MEL28 was comparable whether the T cells had been previously stimulated by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M} (Fig. 6).

Discussion

Xenogeneic fibroblasts expressing retrovirally transduced HLA class I-peptide complexes along with CD80, CD54, and CD58 efficiently stimulate peripheral blood T cells of donors sharing the same HLA molecule. The AAPCs express a human tripartite complex comprising one HLA molecule, human β$_2$-microglobulin, and one encoded peptide. The total yield of CD8+ T cells obtained by stimulation with AAPCs is higher than that achieved with peptide-pulsed autologous dendritic cells, albeit under distinct culture conditions. Several factors may contribute to the high efficiency of the AAPCs. The level of cell surface expression of HLA A2.1, CD80, CD54, and CD58 is elevated, comparable to that of mature primary HLA A2.1+ DCs. The density of the specific HLA–peptide complex may also play an important role. Artificial APCs endogenously express under selective pressure the relevant peptide, which is targeted to the endoplasmic reticulum where peptides are loaded onto nascent HLA class I complexes[22,23]. Expression of the specific complex is therefore maintained irrespectively of the turnover of these complexes at the cell membrane, which is not the case with peptide-pulsed APCs, including artificial APCs derived from *Drosophila* cells[24]. Another advantage of using mouse fibroblasts com-

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Table 1. Expansion and antigen specificity of CD8⁺ T cells after one or two AAPC stimulation[a]

	$AAPC^{A2}$		$AAPC^{A26}$		$AAPC^{A24}$	
	A	B	A	B	A	B
Donor 1						
$CD8^+$ T-cell number[b]	2	25	0.3	8	0.45	9
Specific cytotoxicity	40	98	3	7.5	10	60
Donor 2						
$CD8^+$ T-cell number[b]	5	80	0.9	22.5	0.8	30.5
Specific cytotoxicity	85	96	5	35	10	75
Donor 3						
$CD8^+$ T-cell number[b]	2.9	39.2	1.2	11.1	0.9	6.1
Specific cytotoxicity	80	99	1	45	25	80

[a]Between 8 and 18 million HLA A2.1⁺ donor T cells were plated on the different AAPCs on day 0. Cells were counted and stained for CD8, CD25, CD83, and HLA DR expression after the first (A) and second (B) stimulations. [b]Cell numbers correspond to a starting number of 1 × 10^8 $CD8^+$ T cells. Specific cytotoxicity measured against T2 cells pulsed with the immunizing peptide (as in Figs 4 and 6) is shown as the 10:1 E:T ratio. Background activity measured at the same ratio against an irrelevant peptide (as in Figs 4 and 6) was subtracted.

pared to *Drosophila* cells is their stability in culture and ease of manipulation. The low ability of fibroblasts to process and load peptides onto MHC molecules, as compared to professional APCs, may also contribute to enhance the expression of the specific HLA–peptide complex by decreasing simultaneous presentation of irrelevant peptides[35,36]. Furthermore, primary APCs, like DCs, express six HLA class I alleles and concomitantly present a greater diversity of HLA–peptide complexes. Cytotoxic T lymphocytes of either HLA–peptide specificities are therefore stimulated. In contrast, AAPCs express a single HLA class I molecule efficiently loaded with the relevant peptide.

Vigorous CTL responses were induced against two peptides expressed in melanoma, one derived from the MART-1 and the other from the gp100 antigen. After two rounds of T-cell stimulation, specific CTLs were induced in three out of three donors for MART-1 and two out of three for gp100. These findings are concordant with studies in melanoma patients and normal donors, suggesting that MART-1 elicits a greater immune response than $gp100^{209-2M}$. These results demonstrate that AAPCs can induce strong responses against autoantigens and suggest that they do not only recall primed CTLs—as is the case for the flu response—but also activate naive T cells present at a very low frequency in the peripheral blood of healthy donors. T cells induced by AAPCs against autoantigens specifically kill tumor cells that overexpress these antigens in an HLA class I-restricted manner. This strongly suggests that AAPCs may be used to expand CTLs for clinical purposes. Artificial APCs are stably transduced and thus obviate the need to generate autologous primary cells to effectively induce populations of antigen-specific T cells for each patient. Artificial APCs can easily be generated for different MHC–peptide combinations, and could be modified to stimulate T helper cells if MHC class II–peptide complexes are expressed. Additional costimulatory and/or adhesion molecules may further augment their capacity to promote the expansion of antigen-specific T-cell populations.

For adoptive immunotherapy using antigen-specific T cells, cell doses in the range of 10^9 are typically infused[1–5]. Based on a conservative estimation of 8-fold expansion obtained with $AAPC^{A2}$ or $AAPC^{24}$ after two stimulations (Table 1), generation of 10^9 $CD8^+$ T cells would require about 1.2 × 10^8 peripheral blood $CD8^+$ T cells as the starting material, thus requiring 250–500 ml of blood. If additional cells were needed or if the starting cell number was less, a third round of stimulation or further nonspecific activation using, for example, beads coated with anti-CD3 and anti-CD28 antibodies[28] could be envisaged. Currently, virally infected B cells or DCs can be used to generate T cells for adoptive cell therapies[1–5,31,32]. Transduced mouse fibroblasts provide an alternative cellular system that is very effective in activating B lymphoma cells[33], restimulating genetically modified T cells[34,35], or activating and expanding human primary T cells as shown here. The use of viral vectors should facilitate the generation of AAPCs for other HLA molecules and peptides, starting from other cell types if necessary. Artificial APCs are therefore versatile and useful to study T-cell activation and to induce antigen-specific T cells for clinical purposes.

Experimental protocol

Vector construction. cDNAs were cloned into the *Nco*I and *Bam*HI sites of the SFG vector backbone[36]. A dicistronic vector encoding neomycin phosphotransferase 3′ of the encephalomyocarditis virus internal ribosomal entry site[37] was constructed to express HLA A2.1 (kind gift of Drs. S.Y. Young and N. Cerebi). A dicistronic vector encoding puromycin-*N*-acetyltransferase was used for the minigenes encoding the different peptides used in this study. The human CD8α leader was fused to the peptide antigens to target the endoplasmic reticulum. Monoclonality vectors were constructed for the human β$_2$-microglobulin (kind gift of Dr. S.Y. Young), CD80 (ref. 35), CD54, and CD58 (kind gift of Dr. M. Dustin).

Gene transfer procedures. 293GPG packaging cells[38] were transfected with each plasmid by calcium chloride method as described[39]. A total of 5 × 10^6 NIH 3T3 cells (ATCC, Manassas, VA) were plated in a 6 cm plate and cultured in Dulbecco's modified Eagle medium (DMEM; Mediatech, Herndon, VA) with 10% heat-inactivated donor calf serum (DCS; Hyclone, Logan, UT), penicillin at 100 U ml^{-1}, and streptomycin at 100 μg ml^{-1}. They were infected the day after with cell-free retroviral supernatant (0.45 μm filtration, Acrodisc; Pall Corporation, Ann Arbor, MI) in the presence of polybrene (Sigma, St. Louis, MO) at 8 μg ml^{-1} for 16 h. Geneticin (Sigma) was added at 1.2 mg ml^{-1} to the medium for two weeks to select the cells expressing the A1. Puromycin (Sigma) was added at 3 μg ml^{-1} to the medium for one week to select cells expressing the vector-encoded peptide. After transduction with a monoclonstronic vector, if gene transfer was extremely efficient (>95%), no cell purification was required. If gene transfer was less efficient, transduced cells were purified by using magnetic beads (Dynal, Oslo, Norway) or flow cytometry (Becton Dickinson, San Jose, CA).

Generation of dendritic cells and T-cell purification. Peripheral blood was obtained from normal HLA A2.1⁺ donors in heparinized tubes. HLA typing was performed by PCR in the HLA laboratory at MSKCC. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on lymphocyte separation medium (Accurate Chemical & Scientific Corporation, Westbury, NY). Dendritic cells were generated as described[24]. Briefly, the T-cell-depleted (ER^-) population was prepared by rosetting with sheep red blood cells (Colorado Serum Company, Denver, CO) as described[40]. Two million ER^- cells were plated per well in six-well plates. Granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA) and interleukin-4 (R&D Systems, Minneapolis, MN) were added at 1,000 U ml^{-1} every second day for eight days. Conditioned medium (CM) was prepared by adding 50 × 10^6 ER^- cells on Petri dishes coated with human γ-globulins (Sigma) at 10 mg ml^{-1}. Nonadherent cells were removed and the CM, collected after 24 h, was added (a half or a third of the final volume) to the cells for four days to get fully mature DCs. After four days with CM, the cells had a phenotype of fully mature DCs: they had lost the expression of CD14, expressed high levels of CD40, CD80, MHC class I and class II molecules, and had acquired the expression of the specific marker CD83 (data not shown). T cells were purified as described[40]. Briefly, the T-cell-enriched (ER^+) population was collected from the same donors. After lysis of the sheep red blood cells and three washes in phosphate-buffered saline (PBS) with 2% heat-inactivated fetal calf serum (FCS, Hyclone), B cells, natural killer cells, monocytes-macrophages, and activated T cells were depleted. This was accomplished by incubating cells with mouse IgG monoclonal antibodies directed against CD11b, CD16, and HLA DR DQ, DR (Pharmingen, San Diego, CA) at 1 μg per million cells for 30 min, followed by a panning on Petri dishes coated with goat anti-mouse IgG (Caltag, Burlingame, CA) as described[41]. After three washes in PBS with 2% FCS, the T cells were resuspended at a final concentration of 10 million cells/ml. Dendritic cells were maintained in RPMI 1640 (Mediatech) with 10% FCS. T lymphocytes were maintained in AIM V medium (Life Technologies, Rockville, MD) without serum. Penicillin at 100 U ml^{-1} and streptomycin at 100 μg ml^{-1} were added to all the cultures.

Flow cytometry analysis. To analyze the phenotype of the AAPCs, we used antibodies against human β$_2$-microglobulin, A2.1 (kind gifts of Dr. S.Y. Young), B7.1 (Pharmingen), ICAM-1, and LFA-3 (Becton Dickinson). Anti-CD14, CD80, CD40, HLA DR (Becton Dickinson), and anti-CD83 (Immunex, Marseilles, France) antibodies were used to evaluate the level of

maturation of the DCs. To verify the purity of the preparations of T cells and to study the phenotype of these T cells, we stained cells with antibodies anti-CD19, CD14, CD56, CD16, CD3, CD4, CD8, CD25, CD69, and HLA DR (Becton Dickinson).

Stimulation of specific CTLs. Dendritic cells were pulsed with the peptide (10 μM) for 2 h at room temperature in RPMI without serum. Coculture with T cells was established at the ratio 10 T lymphocytes to 1 DC in 24-well plates, with 1 million T cells per well for 8–10 days, in RPMI with 10% FCS. Artificial APCs were irradiated (1,500 Gy) and plated the day before in 24-well plates at the concentration 10^5 cells/ml in AIM V medium with 5% DCS, 500 μl per well. T cells were resuspended in AIM V medium at the concentration of 2×10^6 cells/ml, added to AAPCs at 500 μl per well, and cultured for 8–10 days. Interleukin-2 (IL-2) (Chiron, St. Louis, MO) was added to the cultures after seven days (20 IU ml^{-1}, every third day). To restimulate the T cells 10–14 days after induction, T cells were cocultured with AAPCs following the same procedure, with 10^5 T cells per well for 10–14 days. Every third day, IL-2 at 20 IU ml^{-1} were added.

Cytotoxicity assays. Standard chromium release assays were performed, using as target cells Transfer associated with antigen processing (TAP) protein-deficient HLA A2.1+ T2 cells (kind gift of Dr. J.W. Young), loaded with the different peptides (10 μM, 1 h at room temperature, in RPMI without serum) before pulsing with ^{51}Cr for 1 h at 37°C. We used 5,000 T2 cells per well in 96 V-bottom plates at different effector:target cell (E/T) ratios for 4 h. We also used SK-MEL-23 and SK-MEL-28 cells as targets (kind gifts of Dr. P. Chapman). They are, respectively, HLA A2.1+ and HLA A2.1+ melanoma cell lines that express MART-1 and gp100 antigens[21]. SK-MEL cells were pulsed with ^{51}Cr as for the T2 cells. We performed 16-h cytotoxicity assays with 1,000 target cells per well. Specific ^{51}Cr release was calculated using the formula ((^{51}Cr release − spontaneous release)/(maximum release − spontaneous release)) × 100. Lytic units (LU) were calculated according to equation (12) in Brynt and coworkers[22].

Peptide synthesis. All the peptides were synthesized in the Peptide Synthesis Facility at MSKCC, resuspended in 50% (vol/vol) RPMI–dimethylsulfoxide (Sigma), and stored at -20°C. The following peptides were used in this study: the influenza matrix protein-derived peptide$_{58-66}$ GILGFVFTL (the peptide); the MART-1 protein-derived peptides$_{27-35}$ AAGIGILTV; the gp100-modified peptides$_{209-217}$ IMDQVPFSV (g209-2M), which efficiently induces CTLs against the natural gp100 peptide[23].

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Effects of CD28 Costimulation on Long-Term Proliferation of CD4$^+$ T Cells in the Absence of Exogenous Feeder Cells[1]

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In this report, conditions for prolonged in vitro proliferation of polyclonal adult CD4$^+$ T cells via stimulation with immobilized anti-CD3 plus anti-CD28 have been established. CD4$^+$ cells maintained exponential growth for more than 60 days during which a total 10^9- to 10^{11}-fold expansion occurred. Cell cultures exhibited cyclical changes in cell volume, indicating that, in terms of proliferative rate, cells do not have to rest before restimulation. Indeed, electronic cell size analysis was the most reliable method to determine when to restimulate with additional immobilized mAb. The initial ~10^5-fold expansion was autocrine, occurring in the absence of exogenous cytokines or feeder cells. Addition of recombinant human IL-2 after the initial autocrine expansion resulted in continued exponential proliferation. Phorbol ester plus ionomycin also induced long-term growth when combined with anti-CD28 stimulation. Analysis of the T cell repertoire after prolonged expansion revealed a diverse repertoire as assessed by anti-TCR Vβ Abs or a PCR-based assay. Cytokines produced were consistent with maintenance of both Th1 and Th2 phenotypes; however, the mode of CD3 and CD28 stimulation could influence the cytokine secretion pattern. When anti-CD3 and anti-CD28 were immobilized on the same surface, ELISAs on culture supernatants revealed a pattern consistent with Th1 secretion. Northern analysis revealed that cytokine gene expression remained inducible. Spontaneous growth or cell transformation was not observed in more than 100 experiments. Together, these observations may have implications for gene therapy and adoptive immunotherapy. Furthermore, these culture conditions establish a model to study the finite lifespan of mature T lymphocytes. *The Journal of Immunology*, **1997, 159: 5921–5930.**

In vivo, T lymphocytes require at least two signals for complete activation. In addition to an Ag signal, a second costimulatory signal allows a T helper cell to produce sufficient IL-2 and other cytokines to allow autocrine-driven clonal expansion (1, 2). The CD28 receptor on T cells is able to provide such a costimulatory signal following interaction with CD80 or CD86 on APCs (2–4). CD28 stimulation has been shown to stabilize cytokine mRNA and can also induce the cell survival gene Bcl-X_L (5). Indeed, the antiapoptotic effect appears to be a major role for CD28 ligation in vivo (6). Recently, the CCR1, CCR2, and CCR5 β chemokine receptors have been identified as the first described genes that are down-regulated following CD28 stimulation (7, 8).

Besides CD28, CD80 and CD86 are also able to bind to another related receptor on T cells, CTLA4, with a higher affinity than for CD28 (9–11). Recent evidence has indicated that the role of CD152 (CTLA4) may be to deliver a negative signal (12) to an activated cell, resulting in anergy or cell death (13), which may be important in clearing cells from the site of an immune response (14). Further, mice deficient of CTLA4 die of massive T cell proliferation within a few weeks of birth (15, 16). Therefore, the relative expression of CD28 or CTLA4 on the surface of a T cell may determine whether the cell will become activated and continue to divide, become tolerant, or die.

The control of T cell homeostasis is complex and not completely understood. The lifespan of normal T cells is heterogeneous, as T cells have been shown to consist of multiple subsets of cells composed of both long-lived and short-lived cells (17). In humans receiving radiotherapy for nonlymphoid malignancies, the intermitotic survival time of naïve T cells is estimated to be about 3.5 years and for memory T cells, about 22 wk (18). To begin to gain an understanding of post-thymic T cell survival, it is necessary to know the replicative capacity of T cells as well as the intermitotic survival time. Because of the ability of CD28 to induce the cell survival gene Bcl-X_L (5), we began studies directed at developing a culture system that would allow the polyclonal proliferation of purified CD4$^+$ T cells. We thus developed a method to stimulate CD4$^+$ T cells that may provide for more optimal CD28 stimulation, with less "feeder cells" expressing B7-1 or B7-2 that might trigger CTLA4. Signal 1 is provided by a mAb directed against CD3, and the costimulatory signal is provided by a mAb directed against CD28 that does not cross-react with CTLA4. In addition, this system allows for long-term polyclonal expansion of HIV-infected CD4 cells (19) due to down-regulation of HIV coreceptors (7). We report that mixtures of adult naïve and memory CD4 cells can undergo about 30 to 40 population doublings (PDs).[3] The cells

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retain a highly diverse TCR repertoire and can be induced to secrete mixtures of cytokines characteristic of Th1 or Th2 cells, depending on the form of CD28 stimulation. This system may be useful in adoptive immunotherapy and gene therapy protocols, not only for viral infections, but also for the treatment of malignancies in which the rapid expansion of purified T cells is desirable.

Materials and Methods

Antibodies

For cell purifications, the following purified and azide-free mAbs were used: anti-CD8 OKT8 (IgG2a), anti-CD11b OKM1 (IgG2b), anti-CD14 63D3 (IgG1), anti-CD16 3G8 (IgG1), anti-CD20 1F5 (IgG2a), and anti-HLA-DR 2.06 (IgG1). All of the hybridomas were obtained from the American Type Culture Collection (Rockville, MD) except 3G8, which was a kind gift of Dr. Stephen Shaw (National Institutes of Health, Bethesda, MD). For stimulations, anti-CD3 OKT3 (IgG1) and anti-CD28 9.3 (IgG2a) were used.

Cells

PBLs were isolated by Percoll gradient centrifugation from leukopacks obtained by apheresis of healthy donors. $CD28^+CD4^+$ T cells were purified as described previously (20) by a negative selection method using magnetic beads (Dynal, Lake Success, NY). In each experiment the purity of the separation was monitored by flow cytometry: $CD28^+$ $CD4^+$ T cells were >98% $CD3^+$, >98% $CD28^+$, and <3% $CD8^+$.

Long-term cell cultures

Cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine (BioWhittaker), and 20 mM HEPES (BioWhittaker). OKT3 and 9.3 were bound to magnetic beads (Tosylactivated M-450; Dynal) at ~150 fg per bead (*cis* stimulation). In some experiments, as noted in figure legends, *trans* stimulation was conducted as follows: 1) Dynal beads were coated with anti-CD28 mAb 9.3 or anti-CD3 mAb OKT3, and equal mixtures of anti-CD3 and anti-CD28 beads added to cells; 2) for plastic-immobilized anti-CD3 stimulation, OKT3 was precoated on the culture wells or flask by overnight incubation with a 10 μg/ml solution. The culture wells or flasks were washed extensively with PBS before use and anti-CD28 stimulation performed by addition of 9.3 mAb at 1 μg/ml. Dynal beads were added to T cells at one to three beads per cell with the cells at 1×10^6 cells per/ml in complete medium. The cultures were fed at 2- to 3-day intervals to maintain a concentration of 1 to 2×10^6 cells/ml. Beads were not removed from culture, but were diluted progressively until restimulation. The cell cultures were counted and monitored for cell size or volume on a Coulter Counter model ZM and Channelyzer model 256 (Coulter, Hialeah, FL) equipped with a 70-μ long-bore orifice tube and restimulated with additional anti-CD3/anti-CD28-coated beads when the volume of the T cell blasts decreased to <400 femtoliters. For unstimulated cells, a lower gate was set at 25.5 femtoliters and for activated cells, a lower gate was set at 76.5 femtoliters so that paramagnetic beads would not be counted along with cells. Cell counts were determined from the total particles above these gates, and viability as assayed by trypan blue dye exclusion was routinely >95%. No exogenous cytokines or feeder cells were added to the cultures. Recombinant human IL-2 was added to certain cultures as indicated, but only after the cells did not respond to the addition of fresh anti-CD3/anti-CD28-coated beads by an increase in cell volume several days after the initial stimulation. PD time was determined by analysis of the exponential phase of cell growth by linear regression using statistical functions in SigmaPlot 3.0 and the formula PD in h = 24 × Log2/B1 where B1 = slope of the line number of cells plotted against days.

CDR3 size analysis to determine TCR Vβ diversity

RNA was extracted from 1×10^7 purified $CD4^+$ cells using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized with the SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies) according to the manufacturer's instructions using 5 μg of total RNA, oligo(dT) 12-18 (0.5 μg/μl), and Superscript II reverse transcriptase. The reaction was incubated at 42°C for 5 min, reverse transcriptase added with an additional 50-min incubation, and the reaction terminated at 70°C for 15 min. Residual RNA was removed by incubation with RNaseH at 37°C for 20 min.

CDR3 length of the variable β region was determined using PCR amplification originally described by Pannetier (21–23). cDNA from purified $CD4^+$ cells was amplified using Vβ region primers for each of 20 Vβ families and a Cβ region (24). Each PCR consisted of 1× Promega (Madison, WI) 10× buffer, 0.2 mM dNTP, 1.7 mM $MgCl_2$, 0.6 μM Cβ primer, 18 μg cDNA, and 0.5 U *Taq* coupled with TaqStart Ab (Clontech, Palo Alto, CA). The reaction was conducted in a 9600 Perkin-Elmer thermocycler (Norwalk, CT) for 40 cycles: 90°C for 25 s followed by 40 cycles of 90°C for 25 s, 60°C for 45 s, 72°C for 45 s, followed by an extension period at 72°C for 5 min. A 2-μl aliquot of this reaction was then submitted to a one-cycle runoff reaction using a 6-FAM-labeled Cβ primer. The samples were then mixed with deionized formamide, Rox-500 size standard (Perkin-Elmer), and dextran blue and subjected to electrophoresis on a 373 Applied Biosystem Sequencer (Foster City, CA) using a 24-cm well to read plates and an 8 M urea, 6% polyacrylamide gel. Data were then analyzed using the GeneScan Software 672 Analysis Software (Perkin-Elmer).

Cytokine assays

Cytokine contents in long-term cultures were determined upon restimulation by washing the cells in fresh medium, restimulation with beads, and collection of supernatants after 24 h. Concentrations of cytokines in cell-free supernatants were assayed by ELISA using commercially available kits obtained from the following sources: IL-2, T Cell Diagnostics/Endogen (Woburn, MA); IL-4, R&D Systems (Minneapolis, MN) or Dianova (Hamburg, Germany); IL-5, TNF-α, granulocyte macrophage-CSF, MIP-1α, MIP-1β, RANTES, R&D Systems; IL-10, Dianova; IL-13, Biosource (Camarillo, CA); IFN-γ, Endogen (Woburn, MA) or R&D Systems. All values reported were assessed by using dilutions of culture supernatant that yielded read-outs within the linear portion of the standard curve.

Results

CD28 costimulation specifically augments long-term $CD4^+$ T cell proliferation

Previous studies have shown that CD28 costimulation can specifically augment the proliferation of T cells during short-term culture. To more fully characterize the proliferative potential of adult $CD4^+$ T cells, cells were initially incubated with either anti-CD3 + anti-CD28 coimmobilized on beads, PHA + recombinant human IL-2 or anti-CD3 immobilized on beads plus recombinant human IL-2, as shown in Figure 1A. In earlier experiments, the ratio of anti-CD3 (OKT3) to anti-CD28 (9.3) conjugated to the beads was titrated and optimal long-term proliferation was observed at a ratio of 1:1 (data not shown). Only cells stimulated with the combination of anti-CD3 and anti-CD28 exhibited long-term growth. Cells cultured with optimal amounts of anti-CD3 plus recombinant human IL-2 or PHA plus recombinant human IL-2 exhibited similar growth rates for the initial 2 to 3 wk of culture compared with anti-CD3 plus anti-CD28-stimulated cells. However, the CD28-costimulated cells remained in exponential proliferation and the anti-CD3 plus recombinant human IL-2 and PHA plus recombinant human IL-2-stimulated cultures entered the plateau phase of growth in the 2nd to 3rd weeks of culture. Cell numbers in the experiment shown in Figure 1A had increased ~1 $\log_{10}$ more in cultures stimulated with anti-CD3 plus anti-CD28 than cultures stimulated with anti-CD3 + recombinant human IL-2 or PHA + recombinant human IL-2 by day 20 of culture. The input cells were typically >97% $CD28^+$, >95% $CD4^+$ (data not shown). It is important to note that APC were removed by negative immunomagnetic selection before the initiation of the culture. This was assured by the failure of the $CD4^+$ cells to grow in the presence of PHA alone. Furthermore, the proliferation induced by anti-CD3/CD28-coated beads was entirely autocrine, as exogenous cytokines or feeder cells were not added to the culture. Finally, the ability of anti-CD28 to sustain cell proliferation was specific, as beads coated with anti-CD3 and a variety of other Abs to T cell surface structures such as MHC class I, CD4, CD5, CD7, CD43, CD45, CD40L, and CTLA4 did not exhibit sustained proliferation (25, and data not shown). Thus, increased cell adhesion to the beads cannot account for specific enhancement of growth by CD28.

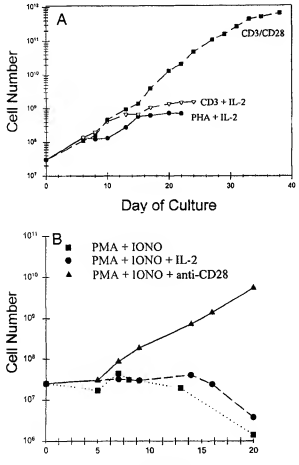

FIGURE 1. CD28 costimulation mediates growth of peripheral blood $CD4^+CD28^+$ T cells in the absence of exogenous cytokines or feeder cells and does not require Ag receptor stimulation. *A*, Anti-CD3 (OKT3) + anti-CD28 (9.3)-coated Dynal beads (solid squares), or anti-CD3 (OKT3)-coated Dynal beads + recombinant human IL-2 100 U/ml (open triangles), or PHA 5 μg/ml + recombinant human IL-2 100 U/ml (solid circles) were added to $CD4^+$ T cells. *B*, PMA 1.9 nM + ionomycin 0.08 μg/ml (solid squares), or PMA 1.9 nM + ionomycin 0.08 μg/ml + recombinant human IL-2 100 U/ml (solid circles), or PMA 1.9 nM + ionomycin 0.08 μg/ml + anti-CD28 (9.3)-coated Dynal beads (solid triangles) were added to $CD4^+$ T cells. Fresh medium was added to the cultures as required and excess cells discarded as described in *Materials and Methods*. Where indicated, recombinant human IL-2 was added to media for cells grown in recombinant human IL-2 to maintain a concentration of 100 U/ml. Cell number was determined using the average of two counts on a Coulter Counter ZM. The total number of cells that would be expected to accumulate is displayed, taking into account discarded cells.

To further delineate the specificity of anti-CD28 to induce long-term autocrine proliferation, $CD4^+$ T cells were stimulated with either PMA plus ionomycin. PMA plus ionomycin plus recombinant human IL-2, or PMA plus ionomycin plus anti-CD28 as shown in Figure 1*B*. Earlier experiments established optimal concentrations of PMA and ionomycin for T cell proliferation (data not shown). The addition of anti-CD28 prolonged and enhanced proliferation observed with PMA + ionomycin. Recombinant human IL-2 could not replace the effect of CD28 addition, similar to the result observed in Figure 1*A* in the context of anti-CD3 stimulation. The inability of recombinant human IL-2 addition to completely replace the growth-promoting effects of CD28 costimulation is likely related to the ability of CD28 and not IL-2 to induce sustained expression of the antiapoptotic gene Bcl-X_L (5). Another possibility is that CD28 may preserve the responsiveness of cells to IL-2 receptor stimulation. Recently, mouse T cell clones first stimulated with Ag and later by cross-linking the TCR were shown to become unresponsive to IL-2 (26). In addition, this experiment indicates that surface engagement of the TCR by anti-CD3 is not required for the growth-potentiating effects of CD28 costimulation, as pharmacologic "bypass" activation appears to be sufficient. Furthermore, a prolonged lag phase was observed in cultures of PMA plus ionomycin plus anti-CD28 stimulation, shown in Figure 1*B*, as revealed by comparing the lag phase in cultures stimulated with anti-CD3 and anti-CD28, shown in Figure 1*A*. The explanation for the increased lag phase in PMA/ionomycin-stimulated cultures is not fully known, but is likely related to the observation that only a subset of cells bearing CD101 responds to anti-CD28 and PMA stimulation, while essentially all $CD28^+$ cells respond when stimulated with anti-CD3 plus anti-CD28 (27). Together, these experiments confirm and extend previous results showing that CD28 delivers a signal that costimulates T cells stimulated by anti-CD3 or by phorbol esters plus calcium ionophore and results in autocrine proliferation for about 1 mo (28).

Cyclical restimulation of $CD4^+$ T cells with immobilized anti-CD3/CD28 results in long-term exponential growth

In the absence of exogenous recombinant human IL-2 addition we have previously shown that $CD4^+$ T cells stimulated with anti-CD3/CD28 will exhibit exponential growth for about 20 days (28), while $CD4^+CD45RA^+$ (naïve) T cells will exhibit exponential growth for about 45 days (29). In addition, we found that repeated addition of beads coated with anti-CD3/CD28 was necessary to sustain cell proliferation, and that addition of exogenous recombinant human IL-2 could further sustain proliferation. In Figure 2*A*, the growth curve of $CD4^+$ T cells cyclically stimulated by anti-CD3/CD28 beads, as described in *Materials and Methods*, is shown. After the fifth restimulation, the cells are no longer able to maintain logarithmic growth and plateau at 8 $\log_{10}$ above the input number of cells, corresponding to a mean population-doubling level (PDL) of 27. In contrast, the period of exponential growth could be extended to >60 days when recombinant human IL-2 was added to the culture. In the experiment shown in Figure 2*A*, the exponential growth phase was extended to a 2×10^{10}-fold increase, or a PDL of 34, with the addition of exogenous recombinant human IL-2 on day 49 of culture, at a point when the cells had become unresponsive to repeated CD3/CD28 stimulation alone. The phenotype of the cells remained >99% TCR-$\alpha\beta^+CD4^+$ (data not shown). The diversity of the TCR expressed in the cells is shown below.

Summarized in Table I are durations of culture and fold expansion of 10 independent $CD4^+$ T cell cultures performed as described in *Materials and Methods*. The cells could be periodically restimulated with anti-CD3/CD28 beads with or without the addition of exogenous recombinant human IL-2, which was added when the culture was unresponsive to further restimulation with anti-CD3/CD28 alone. In the absence of recombinant human IL-2 addition, the cells had an average 2×10^5-fold expansion and an average duration of culture of 39 days. Long-term CD4 proliferation could be obtained with the combination of CD3/CD28 and recombinant human IL-2 stimulation of CD4 cells, with an average duration of proliferation of 82 days. The proliferation was exponential in all cultures as indicated by the semilog plot of cell number vs days of culture. In the cultures grown in the absence of exogenous recombinant human IL-2, the average PD time was

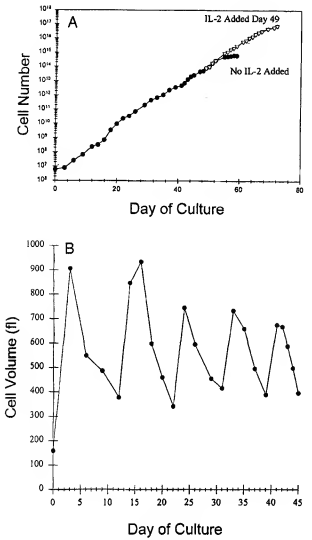

FIGURE 2. Cyclic changes in cell volume occur during long-term exponential proliferation of CD4+ T cells mediated by stimulation with anti-CD3 plus anti-CD28 immobilized on beads. *A*, Growth of CD4+ T cells following six cycles of bead restimulation without the addition of exogenous cytokines (closed circles); the same experiment with the exception of the addition of exogenous recombinant human IL-2 on day 49 (closed circles followed by open triangles). *B*, Mean cell volume (femtoliters) of CD4+ T cells cyclically restimulated with anti-CD3 + anti-CD28-coated beads. At the initiation of the culture, peripheral blood CD4+ T cells had a mean cell volume of 159 femtoliters. Cells were restimulated on days 12, 22, 31, 39, and 45 when the mean cell volume fell below 400 femtoliters. Volume was measured on a Coulter Channelyzer Model C256. For resting day 0 cells, a gate was set to exclude particles <65 femtoliters; for activated cells, a gate was set to exclude particles <200 femtoliters to ensure that beads in the culture were not sized.

37.9 h. In contrast, during the IL-2-dependent phase of cell expansion, the PD time was somewhat slower at 53.2 h. The average PDL for adult CD4+ T cell cultures with exogenous recombinant human IL-2 added was 33 (1×10^{10}-fold expansion). The highest PDL obtained following anti-CD3/CD28 stimulation + recombinant human IL-2 of CD4+ naïve T cells was 41.6 (data not shown). Thus, CD3/CD28 stimulation allows for substantial autocrine-driven proliferation followed by a period of proliferation that is paracrine and dependent upon the addition of exogenous cytokines.

Cyclical restimulation of CD4+ T cells with immobilized anti-CD3/CD28 results in cyclical changes in cell volume independent of proliferative rate

The above results established that exponential proliferation could be maintained for many weeks by periodic restimulation of CD4 cells with anti-CD3/CD28-coated beads. As mentioned earlier, we observed that prolonged exponential proliferation could be achieved if cells were periodically restimulated with Ab-coated beads when cells approached resting cell volumes. In Figure 2*B* the cell volumes are displayed for the growth curve shown in Figure 2*A*. Resting T cells have a mean cell volume of ~170 femtoliters. CD4+ T cells stimulated with immobilized anti-CD3/CD28 increased in volume from 170 femtoliters to nearly 900 femtoliters by 3 days. The cell volume gradually declined over the course of 12 days to 377 femtoliters. At this point, the cells were restimulated by the addition of fresh anti-CD3/CD28 beads and the cell volume again returned to near 900 femtoliters. The culture shown in Figure 2 was restimulated five times, on culture days 12, 22, 31, 39, and 45 before these cells became unresponsive to further restimulation by the addition of anti-CD3/CD28 beads. By comparing Figure 2, *A* and *B*, it is apparent that cyclic changes in cell volume occur that are independent of the rate of cell proliferation. It has been commonly thought that T cell clones need to be "rested" before restimulation. Our results indicate that plateaus in the growth curve are not necessary for cells to regain responsiveness to restimulation. However, a decrease in cell volume appears to serve as an indication that the cells have become responsive to further restimulation. Using this cell stimulation protocol, we have been unable to detect a change in the growth rate of the cells that is related to the readdition of Ab-coated beads. Thus we have concluded that CD4 cell proliferation kinetics can remain exponential and independent of the periodic changes in cell volume. A trivial explanation for the periodic changes in cell volume would be that the beads were included in the cell sizing process, as particles bound to the cell. The beads have a volume that is similar to that of resting cells, and the beads can be electronically gated out from analysis of activated, but not resting, cells. The following observation indicates that the addition of beads does not account for the periodic changes in cell volume, as the beads could be removed from the cell culture on day 3 of culture by magnetic separation, and the beadfree cells were still found to exhibit the cell enlargement and gradual return to near resting volume. Finally, if cells were allowed to grow in culture until their size returned to resting cell volumes, they became unresponsive to restimulation, and cell loss from apoptosis began to occur (data not shown).

Stimulation with immobilized anti-CD3/CD28 of CD4+ T cells results in polyclonal proliferation

To determine whether the cell growth induced by immobilized anti-CD3/CD28 was polyclonal, 10 mAbs directed at 7 different TCR Vβ families covering about 40% of the Vβ repertoire were used to stain cells at the beginning and at day 71 of the culture. As shown in Figure 3, anti-CD3/CD28 stimulation was able to maintain a polyclonal population of CD4+ T cells for 71 days, as all tested Vβ families remained present and there was no variation by more than twofold in the abundance of any given family. The CD4+ T cells shown in Figure 3 are from the same culture shown in Figure 2*B*, so that after 71 days these cells had a mean PDL of 33.2.

Table I. *Summary of proliferative rates of adult CD4+ T cells stimulated by immobilized anti-CD3 plus anti-CD28 for 10 separate experiments*

Exp.	Medium	Duration of Culture (days)	Fold Expansion	Population Doubling Time (hours)	Exponential Growth (days)	Correlation Coefficient
1	No IL-2	33	1.8 E + 04	44	14	0.991
2	No IL-2	34	3.2 E + 05	35	17	0.980
3	No IL-2	40	8.3 E + 04	42	19	0.996
4	No IL-2	44	2.3 E + 05	35	16	0.988
5	No IL-2	44	5.0 E + 05	33	16	0.982
Average		39	2.3 E + 05	38	16	0.987
6	IL-2 Added Day 33	65	8.5 E + 05	59	27	0.995
7	IL-2 Added Day 28	76	5.0 E + 09	41	45	0.994
8	IL-2 Added Day 49	78	4.6 E + 10	48	61	0.996
9	IL-2 Added Day 31	84	4.4 E + 08	64	69	0.997
10	IL-2 Added Day 41	106	4.1 E + 08	54	33	0.993
Average		82	1.0 E + 10	53	47	0.995

Cells were stimulated in the absence of feeder cells as indicated in *Materials and Methods*, and for experiments 1–5 cells were grown for the duration of the culture without the addition of exogenous cytokines. For experiments 6–10 cells were grown without the addition of exogenous cytokines until recombinant human IL-2 was added on the indicated day, as exemplified in Figure 2A. The proliferative capacity was not assessed in experiments 1–5 as the cultures were terminated while cells remained in exponential growth, whereas experiments 6–10 were terminated when cell cultures began to display a plateau phase. Population doubling (PD) time was determined by analysis of the exponential phase of cell growth by linear regression as described in *Materials and Methods*. The cultures had exponential growth as indicated by the goodness of fit of the growth curve with a straight line. The duration of exponential proliferation is indicated.

To further analyze the effect of prolonged CD4 cell culture on the TCR repertoire, a PCR approach based upon measurement of the length of CDR3 was used. CD4 cells were cultured for 60 days in the presence of anti-CD3 and CD28-coated beads and the TCR clonotypes of 20 Vβ families determined by RNA PCR analysis (22, 30). The PCR products were analyzed on an automated DNA sequencer and the intensity of the bands depicted on plots with the heights corresponding to the intensity of the fluorescent bands. The Vβ repertoire as assessed at the start of culture was diverse as each Vβ family had a gaussian distribution pattern of an average of eight peaks separated by three nucleotides (Fig. 4). Visual inspection after 60 days of culture indicated that a less heterogeneous profile of CDR3 sizes was present. About 75% of the Vβ families remained with diverse profiles while the remaining showed clonal peaks that suggested oligoclonal expansions. Thus, prolonged culture of CD4 cells with anti-CD3 and CD28 can result in the clonal contraction or expansion that is not apparent with FACS analysis. However, this high resolution analysis indicates that the culture retains much of the input diversity.

Cytokine mRNA and protein is more abundant in CD4+ T cells stimulated with anti CD3 + anti-CD28 than with anti-CD3 + recombinant human IL-2

It has previously been shown that CD28 stimulation results in increased cytokine production through stabilization of cytokine mRNA as well as through increased de novo synthesis (31). Figure 5 shows that the induction of IL-2 and TNF-α mRNA following anti-CD3/CD28 stimulation is much more pronounced and prolonged than mRNA induced following anti-CD3 + recombinant human IL-2 stimulation. The enhanced cytokine expression did not reflect differences in growth rate, as the cell proliferation was equivalent for the first several weeks of culture, as shown in Figure 1. Cytokine gene expression was not constitutive, as mRNA for IL-2 and TNF was not detected on day 8 before restimulation. However, inducible cytokine mRNA expression was observed on restimulation. To determine whether this increase in cytokine mRNA correlated with secretion, supernatants from $CD4^+$ T cells stimulated with anti-CD3 beads + recombinant human IL-2 or anti-CD3/CD28 beads were collected after 24 h and assayed for a variety of cytokines and chemokines by ELISA, as shown in Figure 6. Anti-CD3/CD28 not only induced higher levels of most cytokines by significant amounts, but also induced the secretion of detectable levels of IL-4 and IL-13. The pattern of lymphokine production reflected what has been seen in Th0/Th1 cells (32, 33). Shown in Table II is the level of cytokine contained in supernatants from anti-CD3/CD28-stimulated cells from the CD4 cells shown in Figures 2 and 3. Supernatants were collected 24 h after the first four stimulations with anti-CD3/CD28 beads and corresponding to the day of culture shown in the table. Levels of IL-2 and IFN-γ induced remained high compared with IL-4, indicating the maintenance of a Th0/Th1 phenotype. Earlier studies have shown that long-term culture of T cells with anti-CD3 and anti-CD28 can lead to a population of cells that secretes predominantly Th2-type cytokines (34). However, these studies were performed with anti-CD3 and anti-CD28 in *trans*, that is, with anti-CD3 immobilized on plastic and soluble anti-CD28. If anti-CD3 and anti-CD28 are presented in *cis* (immobilized on the same bead), the present results suggest that a progressive bias toward Th2 cytokine patterns does not occur. These results suggest that the manner in which CD3 and CD28 are stimulated on the surface of T cells results in differential signal strengths or in differential signals generated through the TCR and CD28 that can elicit distinct patterns of cytokine secretion. These findings may prove useful in adoptive immunotherapy approaches where skewing toward a particular cytokine profile is desirable.

Discussion

Conditions have been developed that permit extensive in vitro CD4 cell propagation. Although we are not aware of previous studies documenting long-term (>4 wk) proliferation of polyclonal CD4 cells, anti-CD3 plus anti-CD28 mAbs have been used by others to clone T cells with high efficiency. Riddell and Greenberg (35) cloned CMV-specific $CD8^+$ T cells and maintained these cells for 3 mo in culture, at which time they maintained their Ag-lytic activity. Another study has demonstrated cloning of $CD4^+CD45RO^-$ T cells under feeder cellfree conditions. However, the addition of exogenous cytokines was required (36). CD2 stimulation has also been utilized in concert with CD28 stimulation to induce long-term autocrine proliferation of $CD4^+$ T cell clones (37).

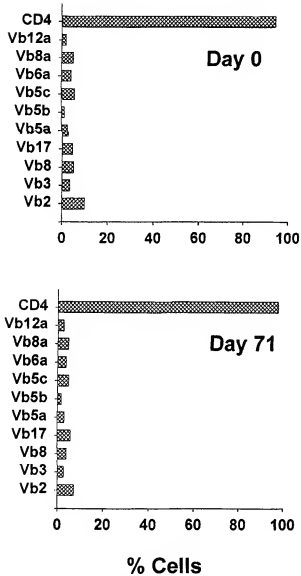

FIGURE 3. Assessment of TCR Vβ repertoire by FACS after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by FACS analysis using mAbs to TCR Vβ families with 10 different mAbs (T Cell Diagnostics and Immunotech) covering 7 different TCR Vβ families as well as an anti-CD4 mAb. Cells from the experiment shown in Figure 2 were stained on the day of initiation of the culture (day 0) and after 71 days in culture. Day 71 of the culture corresponds to a 10 $\log_{10}$-fold expansion or 33.2 mean PDs. The percent positive cells was designated as those with fluorescence intensities greater than 98% of cells stained with phycoerythrin- or FITC-conjugated isotype-specific normal mouse Ig as control. Analysis was performed on a Coulter Epics Elite.

Long-term growth of $CD4^+$ T cells following CD3/CD28 stimulation followed a predictable pattern. Exponential growth was observed throughout the culture. In spite of this the cells exhibited periodic changes in cell volume. The basis for the cyclic changes in cell size remain unknown but may relate to differences of cell cycle distribution. Alternatively, the changes in cell size may indicate a previously unrecognized cell growth function that is regulated by CD3/CD28 stimulation. In support of this, we have noted that cells stimulated in costimulation-deficient conditions, such as with mitogenic lectins and recombinant human IL-2, proliferate at equivalent rates and maintain smaller cell volumes. Cell sizing is useful for determining when to restimulate the cultures, thereby avoiding the plateau in growth curves that occurs with standard culture techniques. This is one factor that is important in avoiding apoptosis and maintaining TCR repertoire during prolonged in vitro culture.

Early in the cultures, cells secreted large amounts of IL-2 and other cytokines, allowing for autocrine proliferation in the absence of added cytokines. However, cells eventually required the addition of exogenous recombinant human IL-2 to continue proliferating. Whether this progression from an autocrine to a paracrine state is a natural state of differentiation of peripheral blood-derived $CD4^+$ T cells or a reflection of the in vitro aging induced by this method of culture is currently under investigation. We have observed that CD28 receptor levels decline when $CD4^+$ T cells were cultured for several weeks (data not shown). The absence of CD28 on the cell surface would mean that a costimulatory signal could not be delivered by anti-CD3/CD28-coated beads and thus would account for the necessity of adding exogenous recombinant human IL-2. In fact, the decline in CD28 expression with age (38) and during progression to AIDS (39–42) is well documented. Following the induction of anergy in either resting or activated T cells, CD28 is down-regulated at the level of mRNA (43). Recently Lloyd et al. (44) showed that IL-4 could down-modulate CD28 expression on $CD8^+$ T cells. These $CD28^-$ cells were found to be less responsive to anti-CD3-mediated proliferation than $CD28^+CD8^+$ T cells.

Immobilizing anti-CD3 and anti-CD28 on beads allowed the titration of the signal delivered through the TCR/CD3 complex with the signal delivered through the CD28 receptor. We found that soluble anti-CD28 did not support long-term proliferation equivalent to immobilized anti-CD28. Preliminary studies suggest that this is due to the ability of immobilized anti-CD28 to maintain CD28 expression, whereas soluble CD28 leads to a more rapid loss of CD28 expression (data not shown). The fact that earlier studies have used soluble anti-CD28 may be the primary reason that long-term polyclonal proliferation of CD4 cells has not previously been reported. Timing, or the delivery, of the signal to the TCR as well as accessory molecules can affect cellular responses such as proliferation and apoptosis (45, 46). For this reason we titrated the relative amounts of anti-CD3 (OKT3) and anti-CD28 (9.3) on beads and found that for long-term growth of $CD4^+$ T cells, a ratio of 1:1 was optimal (data not shown). Similarly we determined the optimal ratio of beads to cells for long-term growth and found that there was no difference between 3 beads per cell and 1 bead per cell, but at 0.3 beads per cell long-term growth was not sustained (data not shown). This signal was independent of CD3 ligation per se, as pharmacologic simulation of CD3 stimulation by phorbol ester and calcium ionophore was also enhanced by the addition of anti-CD28 mAb.

CD28 stimulation delivered a specific signal that enhanced cell growth compared with stimulation with anti-CD3 alone. The initial rates of proliferation were equivalent in CD3 and recombinant human IL-2-stimulated cultures compared with CD3 and CD28-stimulated cultures. However, cells stimulated with optimal amounts of anti-CD3 in the presence of exogenous recombinant human IL-2 did not maintain prolonged growth. These results are in agreement with previous studies in the mouse indicating that Ag-dependent clonal expansion of $CD4^+$ T cells in vivo is dependent on CD28 costimulation (47). This may be due to the ability of CD28 to promote lymphocyte survival in both human and mouse T cells (5, 6).

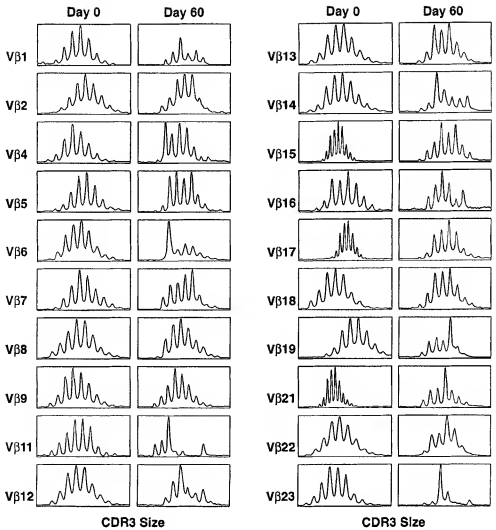

FIGURE 4. Assessment of TCR Vβ repertoire by PCR after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by CDR3 length of the variable β region for 20 Vβ families and a Cβ, as described in *Materials and Methods*. Resting $CD4^+$ T cells isolated from an apheresis product (day 0) and CD3/CD28-stimulated cells on day 60 of culture were analyzed. A gaussian distribution of the eight CDR3 length fragments indicates a polyclonal population, and a nongaussian distribution indicates either an expansion or deletions in particular Vβ subsets within the same family.

The ability to propagate Ag-nonspecific or polyclonal T cell lines first became possible following the identification and characterization of IL-2 as a T cell growth factor (48). However, mixed populations of $CD4^+$ and $CD8^+$ would eventually result in a population of cells that was predominantly $CD8^+$ (49). Furthermore, polyclonal T cell propagation has not been demonstrated with IL-2, possibly due to the ability of IL-2 to prime activated T cells for apoptosis. Subsequent studies of the long-term growth of human T cells have necessitated the use of IL-2 in addition to mitogenic lectins and autologous or irradiated allogeneic feeder cells (37, 50, 51).

Our present studies indicate that polyclonal populations of adult CD4 cells can proliferate for a 10^9- to 10^{11}-fold expansion, equivalent to 30 to 40 PDs. Previous studies by Effros and coworkers have addressed the lifespan of T lymphocytes in vitro (50). They reported a mean PD of 23 ± 7 from adult T cells using lectins and feeder cells for cell propagation. We have consistently achieved higher PDLs. Furthermore, while some reports indicate that rare T cells can grow in vitro for up to 80 PDs (52, 53), our present results indicate that this does not appear to reflect the replicative capacity of the vast majority of polyclonal peripheral T lymphocytes. Thus, our studies indicate that the replicative capacity of CD4 cells is extensive but finite. In separate studies we found heterogeneous replicative properties of CD4 cells as we found that naïve cells had a greater replicative capacity than memory CD4 cells (29). In vitro, costimulation by anti-CD28 appears to have a role in telomerase induction (54); however, it remains to be established whether this has any function in determining the long-term proliferative capacity of the cultures that we observe in vitro or in determining T cell replicative capacity in vivo.

The role of CD28 costimulation in T cell differentiation remains controversial. In mice, CD28 costimulation is required to prime Th1 and Th2 cells; however, CD28 appears to promote the differentiation of cells that secrete Th2 cytokines (55). We have examined cytokine production in cultures following stimulation with beads bound with both anti-CD3 and anti-CD28 (*cis* stimulation) and found a maintenance of a Th1 profile of cytokine production over several rounds of stimulation during

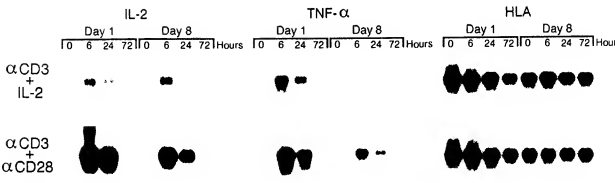

FIGURE 5. Cytokine gene expression remains inducible in cultures of exponentially proliferating $CD4^+$ T cells after anti-CD3 plus anti-CD28 stimulation. $CD4^+CD28^+$ T cells were stimulated and maintained in culture as described in *Materials and Methods*. On day 1 or 8 of the culture, cells were collected and subjected to Northern analysis at 0, 6, 24, and 72 h following stimulation with either anti-CD3 + recombinant human IL-2 100 U/ml or plastic-immobilized anti-CD3 plus anti CD28 1 μg/ml for IL-2, TNF-α, and HLA class I mRNA expression as described in *Materials and Methods*.

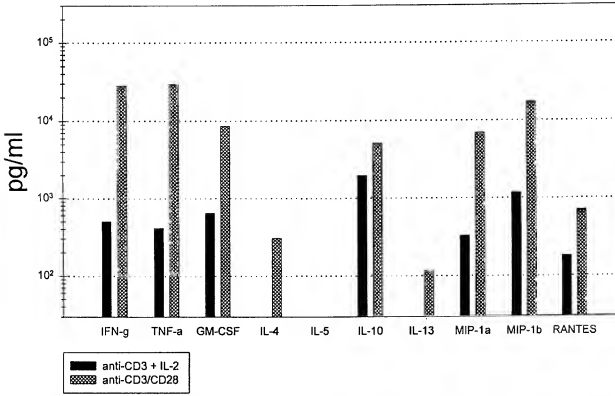

FIGURE 6. Cytokine and β chemokine secretion in cultures of $CD4^+$ cells as measured by ELISA. Anti-CD3- plus anti-CD28-coated beads or anti-CD3-coated beads plus recombinant human IL-2 100 U/ml were added to freshly isolated peripheral blood $CD4^+CD28^+$ T cells at a ratio of three beads per cell. IL-2 as measured by ELISA was 189,600 pg/ml for anti-CD3- plus anti-CD28-coated beads and 40,812 pg/ml for anti-CD3-coated beads plus recombinant human IL-2 100 U/ml. Supernatants for cytokine analysis were collected 24 h later and appropriate dilutions analyzed by ELISA as described in *Materials and Methods*. Sensitivity of the ELISA kits were as follows: IFN-γ, 15.6 pg/ml; TNF-α, 15.6 pg/ml; granulocyte macrophage-CSF, 15.6 pg/ml; IL-4, 15.6 pg/ml; IL-5, 15.6 pg/ml; IL-10, 15.6 pg/ml; IL-13, 19.5 pg/ml; MIP-1α, 31.25 pg/ml; MIP-1β, 31.25 pg/ml; and RANTES, 31.25 pg/ml.

long-term culture. This confirms previous studies that costimulation in *cis* is more efficient than in *trans* (56). Although the mechanism leading to these results remains unknown, we favor the notion that different strengths of Ag dose and costimulation can lead to distinct Th1 and Th2 differentiation. It is also possible that these different forms of stimulation lead to selective survival of Th1- or Th2-like cells, and further studies will be required to distinguish these possibilities. Together, the present results help clarify apparent differences from previous studies of cytokine secretion patterns after CD28 stimulation where repetitive *trans* stimulation could lead to the emergence of a Th2 phenotype (34), while repetitive *cis* stimulation maintained cytokine secretion patterns consistent with a Th1 phenotype (19, 28). Regardless of the mechanism involved, it is possible that these results might be useful to produce polyclonal populations of T cells that have Th1 or Th2 bias.

Table II. *Cytokine secretion from CD4+ T cells after four cycles of re-stimulation with immobilized anti-CD3 plus anti-CD28*

Stimulation	Day of Culture	IL-2	IFN-γ	IL-4
1	1	18,875	2,083	<62.5
2	13	40,682	17,917	173
3	23	19,277	14,229	267
4	32	10,099	6,993	<62.5

Supernatants were collected 24 h after each stimulation. For stimulations 2, 3, and 4, cells were washed out of conditioned media and reseeded into fresh media in order to measure de novo cytokine production. Values shown are in pg/ml. Sensitivity of the ELISA kits were as follows: IL-2, 61 pg/ml; IFN-γ, 40 pg/ml; IL-4, 62.5 pg/ml.

Many lines of evidence point to an impairment of the cellular immune system with increasing age. Engwerda, Handwerger, and Fox (57) have shown that the response of both CD4 and CD8 or naïve and memory cells to CD28 costimulation is impaired in aged mice. However, in aged humans there is a decline in CD28 expression (38), and a recent study has detected clonal expansions within certain TCR Vβ subsets in aged humans (58). One reason for decreased immune function or repertoire in the elderly is thought to be related to the decrease in thymic export of T cells following involution. In a study of $CD4^+$ T cell regeneration following chemotherapy, Mackall et al. (59) found that age or thymic volume correlated with the ability and speed of CD4 cell counts to return to pretreatment levels. Thus, the ability of peripheral T cells to expand ex vivo and reconstitute an impaired immune system has remained in doubt. The data presented here provide further evidence that substantial ex vivo expansion of polyclonal $CD4^+$ T cells is possible following cyclical stimulation with immobilized anti-CD3 and anti-CD28 mAbs. Highly diverse populations of CD4 cells could be maintained for at least 8 wk in cultures. All results are compatible with the notion that this approach leads to physiologic cell growth. Furthermore, in experiments involving cells from more than 100 donors, we have not observed transformation after CD3 and CD28 stimulation. Thus, this approach should permit adoptive immunotherapy and gene therapy strategies for immunodeficiencies and malignancies, as well as facilitate further studies on the replicative capacity of T cells.

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B7-1-dependent co-stimulation results in qualitatively and quantitatively different responses by CD4$^+$ and CD8$^+$ T cells

To characterize better the co-stimulatory activity of native B7-1 in the absence of other receptor/ligand interactions that might contribute to the response, B7-1 was purified by monoclonal antibody (mAb) affinity chromatography. Immobilization of purified B7-1 with anti-T cell receptor (TCR) mAb on cell-sized latex microspheres provided an effective stimulus for activation of both CD4$^+$ and CD8$^+$ T cells as measured by proliferation, development of effector function, and changes in motility and adhesion. The CD4$^+$ T cell response was prolonged and resulted in efficient interleukin-2 production and clonal expansion. In contrast, CD8$^+$ responses were transient. Proliferation and clonal expansion peaked on days 3 and 4, coincident with maximal expression of lytic effector function, and the cells then died. These results demonstrate that B7-1 mediated co-stimulation is sufficient for the induction of effector function in both helper and cytotoxic T cell precursors, but suggest that B7-1 co-stimulation is not sufficient to sustain helper-independent CD8$^+$ CTL responses. When the dose responses of CD4$^+$ and CD8$^+$ T cells to B7-1 were compared, CD8$^+$ T cells were found to require higher densities of B7-1 to attain an equivalent level of activation, suggesting that the level of expression of B7-1 by APC may influence the development of helper or CTL responses. Finally, in contrast to results obtained by others with B7-1 transfectants, purified B7-1 did not provide co-stimulation when presented on a surface separate from the TCR stimulus.

1 Introduction

TCR-mediated antigen-specific T cell signaling is necessary for the induction of T cell activation [1]. However, it has become clear that in many situations this antigen specific signal must be augmented by antigen-nonspecific co-stimulatory signals [2, 3]. These co-stimulatory, or second signals may be provided by soluble factors [4] as well as cell surface molecules [5] that bind to co-stimulatory receptors on the T cell. The B7-1 molecule, present on activated APC, has been shown to be a ligand for the co-stimulatory receptor CD28 that is expressed on essentially all mouse T cells [6–8].

Ligation of CD28 on T cells by the molecule B7-1 [9, 10] or the related homolog B7-2 [11, 12] can drive optimal proliferation and IL-2 production in response to stimulation through the TCR. In addition, it has been found that this interaction is necessary for the prevention of the state of hyporesponsiveness known as anergy that occurs in some CD4$^+$ T cells receiving stimuli only through the TCR [13, 14]. With respect to CD8$^+$ T cell responses, it has been shown that normally nonstimulatory tumors, when transfected with B7-1, can stimulate the induction of CTL activity *in vitro* [15, 16], and *in vivo*, certain tumors not normally rejected by syngeneic hosts may be rejected upon transfection with B7-1. This rejection is independent of CD4$^+$ T cells and results in long-lasting tumor-specific immunity [17–19].

While B7-1 can provide co-stimulatory signals for both CD4$^+$ and CD8$^+$ T cells, a number of observations suggest that there may be differences in the responses of the two subsets to this stimulus. In one study where responses of CD4$^+$ or CD8$^+$ T cells were compared, a relative defect in Ca^{2+} flux and proliferative response after CD28 ligation or B7 co-stimulation was seen in the CD8$^+$ subset [20]. Also, while most studies agree that B7-2 is as good or better than B7-1 for co-stimulation of CD4$^+$ T cells [11, 12, 21–23], conflicting results have emerged regarding the CD8$^+$ subset. In one study, B7-1 and B7-2 were shown to be equally effective for the co-stimulation of CTL induction [24], while in another, B7-1 was shown to be far superior [25]. The normal CD8$^+$ response to LCMV challenge in CD28-deficient mice [26], and the findings that CD8$^+$ T cells express lower levels of CD28 [8] and higher levels of CTLA4 [27, 28] than do CD4$^+$ T cells, also suggest that B7-1 may function differently in co-stimulating CD4$^+$ and CD8$^+$ activation.

In this study, the role of B7-1 co-stimulation in CD8$^+$ and CD4$^+$ activation has been directly compared. The natural CD28 ligand mouse B7-1 has been purified by mAb-affinity chromatography and incorporated onto the surface of microspheres along with anti-TCR mAb. This approach minimizes potential contributions from other receptor/ligand interactions that might play distinct roles in activating CD4$^+$ and CD8$^+$ T cells. In addition, it allows CD4$^+$ and CD8$^+$ T cell responses to be directly compared using

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Abbreviations: DOC: Deoxycholate **MESF:** Mean equivalent soluble fluorochrome **ER:** Endoplasmic reticulum

Key words: B7-1 / Cytotoxic T lymphocyte / Co-stimulation / CD4$^+$ T cell / CD8$^+$ T cell

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an identical stimulus for each subset. The results obtained using this approach demonstrate that B7-1 is sufficient to co-stimulate $CD4^+$ and $CD8^+$ responses, but that the responses differ both qualitatively and quantitatively. The biological implications of these differences are discussed.

2 Materials and methods

2.1 Purification of B7-1

The hybridoma producing the anti-B7-1 mAb 16-10A1 [29] and CHO-B7-1 transfectants expressing native B7-1 on the surface [9] were a generous gift of Hans Reiser (Dana Farber Cancer Institute, Boston, MA). 16-10A1 was purified from culture supernatants on a protein A column, and coupled to Sepharose at 1 mg protein/ml (wet) to prepare a 4-ml affinity column. The column was pre-equilibrated with 25 ml 10 mM phosphate buffer containing 0.5% Triton X-100 prior to sample application.

CHO-B7-1 transfectants were grown in roller bottles, harvested by brief treatment with 0.02% EDTA in PBS, pelleted, and stored at −80°C. For the purification of B7-1, 2×10^{10} cells were thawed rapidly at 37°C, promptly resuspended in lysis buffer (50 mM sodium phosphate pH 7.2 containing 1% Triton X-100, 0.2 mM PMSF and 20 μg/ml aprotinin) at 10^7 cells/ml and incubated for 40 min on ice with periodic agitation. All subsequent purification steps were performed at 4°C. Insoluble material in the lysate was removed by centrifugation at 1000 × g for 20 min followed by ultracentrifugation of the resulting supernatant for 45 min at 100 000 × g. Supernatant was then applied to the 16-10A1 affinity column.

After application of the lysate, the 16-10A1 column was washed consecutively with 20 ml of each of the following buffers: 10 mM sodium phosphate, 0.5% Triton X-100 pH 7.2; 10 mM Tris-HCl, 0.5% Triton X-100 pH 8.0, and Tris-buffered saline pH 8.0 with 0.5% deoxycholate (DOC). B7-1 was eluted using 15 mM sodium carbonate pH 12.0 containing 0.5% DOC and 250 mM NaCl. Fractions of 1 ml were collected into tubes containing 0.075 ml 0.4 M Tris-HCl pH 8.0 and stored at −20°C. All buffers contained 20 μg/ml aprotinin.

Protein determinations were done using the Pierce bicinchoninic acid (BCA) protein assay kit (Rockford, IL) according to manufacturer's instructions. ELISA were performed as described [30] using 16-10A1 as the primary Ab and goat anti-hamster horseradish peroxidase-conjugated Ab (Jackson, West Grove, PA) as the secondary Ab. Peak fractions containing B7-1 serological activity were pooled and stored at −20°C for later use. Purified material was analyzed by SDS-PAGE on 10% acrylamide slab gels using the buffer system of Laemmli [31]. Gels were silver-stained for protein according to the method of Morrissey [32]. The yield of purified protein using this procedure is approximately 0.35 mg/10^{10} CHO-B7-1.

2.2 Microsphere preparation

Sulfate polystyrene latex microspheres (5-μm diameter; Interfacial Dynamics Corp., Portland, OR) were stored at 4°C in distilled water. For immobilization, purified F23.1 mAb [33] was added to microspheres in PBS to yield a suspension at 10^7 particles/ml and 0.5 μg/ml F23.1. This suspension was incubated at 4°C for 1.5 h with continual mixing. An equal volume of 1% endotoxin-free BSA (Sigma, St. Louis, MO) in PBS was then added and mixing was continued for an additional 0.5 h. After blocking with BSA, microspheres were washed three times with RPMI containing 5% newborn bovine serum (NBS) and 10 mM HEPES by centrifugation at 500 × g, and finally resuspended in a convenient volume of complete tissue culture medium.

B7-1 purified as described above was at 150 μg/ml in 0.5% DOC. Class I MHC protein was purified as described [34], and was at 50 μg/ml in 0.5% DOC. The membrane proteins were immobilized by dilution into a suspension of microspheres in PBS, under conditions that gave a final DOC concentration of 0.02% or less. The suspensions were then incubated for 1.5 h, blocked with 1% BSA and washed as described above. Using 1 μg B7-1 per 10^7 microspheres resulted in maximal surface density of B7-1 as determined by flow cytometry (see below). Lower densities were achieved by using a lower starting protein concentration. For the co-immobilization of purified B7-1 or class I Ag with F23.1, microspheres bearing F23.1 were prepared as described above (without BSA blocking) and then washed once or twice with PBS by centrifugation at 1000 × g. Class I MHC K^b or B7-1 was then immobilized on the F23.1-bearing microspheres as described above. All procedures involving the preparation of microspheres for use in culture were carried out under sterile conditions.

2.3 Flow cytometry

F23.1 on microspheres was detected using polyclonal goat anti-mouse FITC-labeled Ab (Jackson) with goat FITC-labeled IgG (Jackson) as a control. B7-1 was detected with the FITC-conjugated rat IgG2a,κ mAb 1G10 using rat FITC-labeled IgG2a,κ as a control. FITC-conjugated Y3 was used to stain K^b (all from PharMingen, San Diego, CA). FITC-labeled anti-CD8α, FITC-labeled anti-CD3 (Caltag, San Francisco, CA), PE-labeled anti-CD4 and FITC-labeled anti-CD45R/B220 (PharMingen) were used to stain purified $CD4^+$ and $CD8^+$ T cell subsets to verify purity. Cells and microspheres were stained using identical protocols. Briefly, 10^5 cells or microspheres were stained with the relevant Ab for 20 min at 4°C in 0.1 ml Hanks' balanced saline with 2% FCS and 0.02% NaN_3 (FACS buffer) in the dark. Cells or microspheres were then washed two times with FACS buffer and resuspended in 0.3 ml FACS buffer for immediate analysis.

Samples were analyzed on a Becton Dickinson FACScan, collecting 10 000 events for cells and 5000 for microspheres. Identical photomultipler tube voltages and logarithmic amplifier settings for the detection of FITC were used for all analyses. Quantitative flow cytometry was performed using FITC standard microbeads (Flow Cytometry Standards Corp., San Juan, PR) essentially as directed by the supplier, based on published principles [35]. Standard microbeads containing four different FITC mean equivalent soluble fluorochrome (MESF) levels, as well as FITC$^-$ microbeads were run immediately before stained micro-

spheres or cells. The mean channel number obtained for each standard microbead preparation was plotted against the MESF values specified by Flow Cytometry Standards Corp., and a best fit curve was produced. The analysis demonstrated that the logarithmic amplifier and system as a whole responded linearly within the range of FITC fluorescence detected on stained samples. MESF were calculated by applying the equation describing the best curve fit to the mean FITC channel number obtained with microspheres stained with specific Ab less that obtained with isotype control. Thus, MESF is a linear representation of the amount of ligand present on the surface. In all experiments, F23.1 density was essentially identical in the presence and absence of co-immobilized ligands.

2.4 T cell activation assays and cell populations

T cells used were from 6–12-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) maintained in the University of Minnesota animal facilities according to NIH guidelines. Lymph nodes were harvested into RPMI containing 5% NBS and 10 mM HEPES and single-cell suspensions were produced with 5–8 strokes of a Dounce homogenizer. Resulting suspensions were treated with 11 mM $KHCO_3$, 152 mM NH_4Cl to remove contaminating RBC, immediately centrifuged, and resuspended at 10^7/ml in complete medium (RPMI 1640 supplemented with 10% FCS, 10 U/ml penicillin, 10 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM Hepes, 2 mM L-glutamine and 50 μM 2-ME). Adherent cells were depleted by incubation of 8 ml of this suspension for 1.5 h at 37°C in 10-cm Falcon (Franklin Lakes, NJ) tissue culture dishes followed by removal of non-adherent cells by gentle swirling and two washes with 8 ml RPMI supplemented with 5% NBS and 10 mM HEPES. Resulting cells were further purified into $CD4^+$ or $CD8^+$ subsets using negative selection on Biotex (Edmonton, Canada) mouse cell enrichment columns according to the manufacturer's instructions. Cells purified in this manner were always > 95% positive for the selected T cell phenotype and were < 1% positive for B220 and the reciprocal T cell phenotype (data not shown).

LPS blasts were prepared by incubation of RBC lysed splenocytes from C57BL/6 mice with 50 μg/ml LPS (*Escherichia coli* 011/B4; Sigma) for 48 h in complete medium at 37°C. Prior to use as co-stimulators, these cells were irradiated with 3000 rad using a cesium gamma source. CHO-B7-1 used as co-stimulators were fixed with paraformaldehyde and washed extensively prior to use, essentially as described [36]. All 37°C cell incubations were performed in a humidified atmosphere of 5% CO_2 and 95% room air.

2.5 Cell culture

Responders were plated in 96-well flat bottom culture plates (Falcon) at 5×10^4 per well. Unless otherwise indicated, 10^5 microspheres were co-cultured in triplicate with responders in a final volume of 0.2 ml complete medium/well; cultures were incubated at 37°C for 48 h except as indicated in Fig. 3 and 4. For assessment of proliferation, cultures were pulsed with 1 μCi $[^3H]$thymidine for 6–8 h, lysed with distilled H_2O, and the amount of $[^3H]$thymidine incorporated into DNA was determined by liquid scintillation counting.

2.6 IL-2 assay and Ab blocking

IL-2 was detected in 0.05 ml supernatants removed from cultures prior to pulsing with $[^3H]$thymidine, using IL-2 ELISA reagents obtained from PharMingen according to the protocol provided by the supplier. Standard curves using recombinant mouse IL-2 (PharMingen) were set up to quantitate IL-2 production. Antibody blocking experiments were performed by pre-incubating microspheres for 20 min at 4°C with 1:200 or 1:50 dilution of culture supernatants from the 16-10A1 or GL1 [37] hybridomas, 5 μg/ml purified BE29G.1 mAb [anti-intercellular adhesion molecule (ICAM)-1, a kind gift from Adrienne A. Brian], or with medium alone prior to plating with responders; the final concentration of Ab in culture was one half that present during the pre-incubation.

2.7 CTL assay

2C TCR-transgenic mice were provided as kind gift from Dennis Loh [38]. Purified $CD8^+$ T cells were prepared as described above from the lymph nodes and spleens of transgenic mice housed under specific pathogen-free conditions. Purified cells were cultured in 2 ml complete medium at a density of 3×10^4 cells and 5×10^5 microspheres per well in 24-well plates. At the times indicated, the cells were collected, centrifuged, and resuspended in medium. Serial dilutions of the cells were plated in 96-well V-bottom plates with 1×10^5 ^{51}Cr-labeled EL4-L^d target cells (a kind give of Victor Engelhard) [39] in a final volume of 200 μl per well. After a 4-h incubation at 37°C, 100 μl supernatant was removed from cultures and ^{51}Cr release was determined. Percent specific ^{51}Cr release was determined by the subtracting spontaneous release from experimental group release and dividing by maximum release minus spontaneous release. Maximum release was determined by treatment with 3% SDS. Immediately prior to harvest for CTL assay, 200 μl aliquots were removed from each 2-ml culture, transferred to 96-well plates and assayed in triplicate for proliferation as described above.

2.8 Microscopy

Photomicrographs were taken using a Zeiss Axiovert microscope with a heated stage. Phase-contrast optics were utilized to facilitate visualization of cells and microspheres. Microspheres 5 μm in diameter were present in cultures to serve as an internal size reference.

3 Results

3.1 Purification of B7-1

Approximately 2×10^{10} CHO transfectants expressing a full-length cDNA clone of B7-1 were grown in roller bottle cultures, and cell lysate was prepared for affinity chroma-

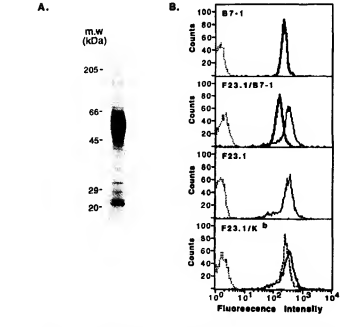

Figure 1. Purification of mouse B7-1 and immobilization on cell-size microspheres. (A) fractions eluted from a 16-10A1 mAb affinity column loaded with CHO-B7-1 lysate were screened for protein content and B7-1 serological activity by ELISA. Peak fractions were pooled and 0.7 μg purified material was analyzed by SDS-PAGE and silver-stained. (B) Microspheres were prepared with the ligands indicated in each panel. Microspheres bearing these immobilized ligands were then stained for flow cytometry to quantitate ligand density. Isotype control (dotted curve); anti-B7-1 (thick curve); anti-mouse IgG, detecting F23.1 (light curve), anti-K^b (dashed curve).

tography as described in Sect. 2.1. Cell lysates were applied to an affinity column prepared with Sepharose coupled to the anti-B7-1 mAb 16-10A1. The fractions obtained upon specific elution were screened, and revealed a single peak in B7-1 serological activity that was coincident with the peak in total protein (data not shown). Analysis of pooled peak fractions by SDS-PAGE revealed a broad band migrating with an apparent molecular mass of about 60 kDa, two faint bands migrating with apparent molecular masses of 33 and 30 kDa, and a 22-kDa band that co-migrated with the light chain of the 16-10A1 mAb (Fig. 1A and data not shown).

The core molecular mass of murine B7-1 based on amino acid sequence is 30 kDa, but glycosylation results in a broadly migrating band of about 60 kDa detected in anti-B7-1 immunoprecipitates [29, 40]. Thus, the major band centered at 60 kDa is mature B7-1, while the 33- and 30-kDa bands are likely to be partially processed endoplasmic reticulum (ER) products recognized by the 16-10A1 mAb. The 22-kDa band was found to be reactive with anti-hamster IgG as indicated by the partial extraction of this contaminant with anti-hamster IgG coupled to Sepharose (data not shown), indicating that it is 16-10A1 light chain leached from the affinity column during elution. Quantitative analysis of Coomassie blue-stained gels indicates that 83% of the staining density is in the broad 60-kDa band, while the bands constituting ER products and light chain represent 5 and 10%, respectively.

3.2 B7-1-dependent co-stimulation of CD4+ and CD8+ T cells results in different responses in the two subsets

The Vβ8-specific anti-TCR mAb F23.1 was immobilized on 5-μm sulfated polystyrene microspheres, either alone, with purified B7-1, or with the purified MHC class I antigen K^b. Fig. 1B demonstrates that the density of F23.1 on microspheres is maintained at a constant level upon co-immobilization with B7-1 or K^b. Thus, it is possible to vary the amount of ligand co-immobilized with F23.1 on microspheres without significantly altering the level of F23.1.

Microsphere preparations characterized by flow cytometry in Fig. 1B were culturd with resting $CD4^+$ or $CD8^+$ T cells. Microspheres coated with B7-1 alone did not stimulate proliferation or IL-2 production, whereas those coated with F23.1 alone had only a marginal effect on proliferation and no IL-2 was detected in cultures of either T cell subset. However, when B7-1 and F23.1 were co-immobilized, a synergistic augmentation of proliferation and IL-2 production was observed in both $CD4^+$ and $CD8^+$ T cells (Fig. 2, solid bars). While B7-1 co-stimulation results in the production of detectable IL-2 in

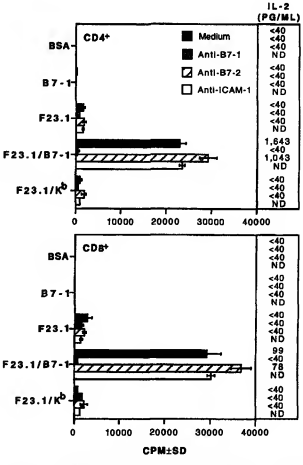

Figure 2. Purified B7-1 co-stimulates $CD4^+$ and $CD8^+$ proliferation and IL-2 production in response to TCR ligation. Purified $CD4^+$ (top) or $CD8^+$ (bottom) T cells (5×10^4) were incubated with the microspheres indicated, and with the additions indicated in the legend. Results represent mean ± SD of triplicate cultures harvested at 48 h. IL-2 determination was performed on supernatants harvested at 42 h; ND indicates not done.

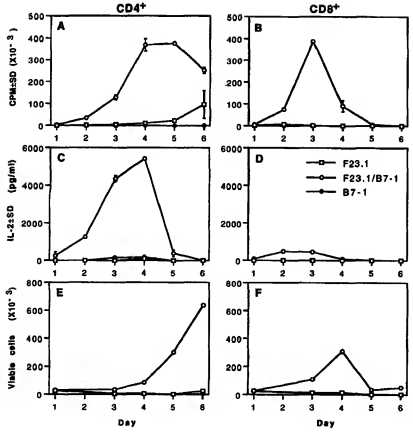

Figure 3. The development of B7-1-dependent responses is different in $CD4^+$ and $CD8^+$ subsets. $CD4^+$ (A, C and E) or $CD8^+$ (B, D and F) T cells (5×10^5) were cultured for the times indicated with microspheres bearing F23.1 alone (□), F23.1 and B7-1 (○) or B7-1 alone (◇). (A, B) Mean cpm ± SD of triplicate cultures pulsed with $[^3H]$thymidine for the last 6-h of culture. (C, D) IL-2 levels ± SD detected in triplicate cultures. (E, F) Viable cell number was determined by trypan blue exclusion; analysis was not performed on day 2.

both T cell subsets, approximately 15-fold more is detected in $CD4^+$ cultures than in $CD8^+$ cultures. Further purification of B7-1 by gel filtration resulted in material that was more than 90% pure based on densitometric analysis; this further-purified B7-1 demonstrated co-stimulatory activity identical to that shown in Fig. 2 (data not shown). Finally, co-immobilization of the syngeneic antigen K^b had no augmenting effect over and above that seen with F23.1 alone. Similar results have been obtained using the anti-CD3 mAb 2C11 as the TCR ligand (data not shown).

Antibody blocking was done to confirm that responses were dependent on B7-1, and to determine whether B7-2 or ICAM-1, both present on T cells, might contribute to the responses. Anti-B7-1 mAb specifically blocked co-stimulation of proliferation and IL-2 production provided by B7-1 co-immobilized with F23.1 (Fig. 2). In contrast, antibody specific for the alternate CD28 ligand B7-2 had no effect on the response (Fig. 2, striped bars). Antibodies against lymphocyte functional antigen (LFA)-1 (not shown) and ICAM-1 (Fig. 2, open bars) also had no inhibitory effect on the B7-1-dependent co-stimulation of T cell responses. In no instance did any of these reagents significantly block the minimal proliferative response observed in the presence of F23.1 alone (Fig. 2). These results demonstrate that in the absence of additional molecular interactions between the TCR ligand-presenting surface and the T cell responder, purified B7-1 is sufficient to co-stimulate $CD8^+$ as well as $CD4^+$ T cell proliferation in response to TCR ligation. They further suggest that additional co-stimulation resulting from cell/cell interactions in the cultures are not likely to contribute to the responses.

The proliferation, IL-2 production, and clonal expansion of $CD8^+$ and $CD4^+$ T cells in response to B7-1 co-stimulation was examined over the course of 6 days (Fig. 3). No clonal expansion or IL-2 production, and only minimal proliferation, occurred in response to F23.1 alone, while B7-1 co-stimulated these responses in both subsets. However the magnitude and time course of these responses differed dramatically in the two subsets. Significant clonal expansion was observed on day 4 in $CD8^+$ cultures co-stimulated with B7-1, and this corresponded with the peak proliferative response and maximal IL-2 production 1 day earlier (Fig. 3B, D and F). After day 3 of culture, $CD8^+$ proliferation and IL-2 production both fell precipitously. Likewise, after day 4, the expanded cells died rapidly, such that by day 6, the number of viable cells in culture was nearly equal to the number present at the initiation of culture. In experiments not shown, the loss of cells observed in $CD8^+$ T cell cultures after day 4 was found to be the result of apoptotic death.

In contrast to the responses of $CD8^+$ T cells to B7-1 co-stimulation, $CD4^+$ responses were sustained (Fig. 3A, C and E); proliferation and expansion continued through day 6. Also in contrast to $CD8^+$ T cells, IL-2 was detectable in culture supernatants through day 5, and was produced at much higher levels. In accord with data in Fig. 2, the B7-1-dependent proliferation of $CD8^+$ T cells on

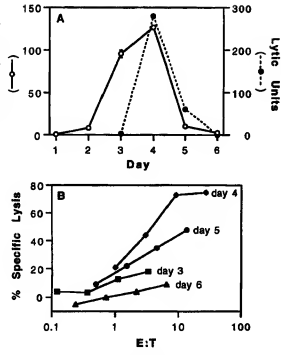

Figure 4. Purified B7-1 co-stimulation results in generation of a helper-independent CTL response. Purified $CD8^+$ 2C transgenic T cells (3×10^4) were cultured in 24-well plates with 5×10^5 microspheres bearing co-immobilized F23.1 and B7-1. Cultures were harvested at the times indicated and assayed for proliferation and CTL activity using EL4-L^d target cells as described in Sect. 2.7. No cytotoxic activity was detected using EL4 cells as targets. (A) Proliferation and cytotoxicity expressed as lytic units. Lytic units were calculated from the data shown in (B). One lytic unit is defined as the number of cells required to lyse 50% of the target cells in the 4-h ^{51}Cr-release assay, and are shown as lytic units per 1×10^3 responder cells. In parallel cultures at all time points, proliferative responses to microspheres having just B7-1 on the surface were less than 200 cpm, and less than 400 cpm with only F23.1 on the microspheres. No cytotoxic activity could be detected for cells cultured with microspheres having only B7-1 or F23.1 on the surface. (B) Percent specific lysis of EL4-L^d targets at different effector : target (E:T) ratios by cells cultured for the indicated number of days in the presence of microspheres having F23.1 and B7-1 co-immobilized on the surface.

days 2 and 3 of culture is higher than that seen in $CD4^+$ cultures (Fig. 4 A and B). Thus, B7-1 co-stimulates both $CD4^+$ and $CD8^+$ T cell activation and expansion, but the subsets differ with respect to the development of these responses; $CD4^+$ responses are more sustained, while $CD8^+$ responses are more rapid and transient.

3.3 B7-1 co-stimulation results in the generation of a helper-independent CTL response

The activity of purified B7-1 in co-stimulating helper-independent induction of cytolytic activity in $CD8^+$ CTL precursors (pCTL) was examined using $CD8^+$ T cells from unprimed 2C TCR transgenic mice on a C57BL/6 background. T cells expressing the 2C TCR are positively selected on K^b in H-2^b mice and the TCR is specific for the L^d alloantigen [38, 41]. When purified $CD8^+$ 2C transgenic T cells were cultured with microspheres bearing F23.1 alone, no proliferation was detected, while co-immobilization of F23.1 with B7-1 resulted in significant proliferation following a time course similar to that seen in wild-type C57BL/6 $CD8^+$ T cell cultures (Fig. 4A and 3B). No cytotoxic activity was detected in cultures stimulated with F23.1 alone, but cells from cultures stimulated with microspheres bearing co-immobilized F23.1 and B7-1 demonstrated potent L^d-specific cytotoxic activity (Fig. 4B). Cytotoxic activity against EL4-L^d is undetectable before day 3, rises rapidly to peak on day 4, and declines thereafter (Fig. 4). Thus, a TCR signal alone is insufficient to induce CTL activity, while B7-1 co-stimulation allows the induction of a helper-independent CTL response in unprimed $CD8^+$ transgenic T cells. This response is independent of $CD4^+$ help and is likely to be independent of interactions between the responding cell and the ligand-bearing surface other than those mediated by immobilized F23.1 and B7-1.

3.4 B7-1 co-stimulation is dependent on the density of F23.1 and B7-1

When F23.1 density on microspheres was titrated without B7-1 co-immobilized, even high densities of F23.1 were unable to induce significant proliferation (Fig. 5A). Proliferation to F23.1 alone in both subsets of T cells was always less than the B7-1-co-stimulated response using the same density of F23.1, except at the lowest density of F23.1 used where F23.1 both with and without co-immobilized B7-1 gave responses equivalent to background (Fig. 5A). In this experiment, it is difficult to compare $CD4^+$ and $CD8^+$ responses because of the different sensitivities of the two T cell subsets to F23.1 levels and because at high densities of F23.1, optimal B7-1 co-immobilization was not achieved. Nonetheless, the results show that in both subsets of T cells, a lack of co-stimulation cannot be overcome by increasing TCR ligand density.

When F23.1 was maintained at a constant density and B7-1 was titrated, it was found that the proliferative response was directly proportional to the amount of B7-1 present on the microspheres (Fig. 5B), but the threshold for detecting B7-1-dependent co-stimulation of proliferation is about twofold higher for $CD8^+$ cells than for $CD4^+$ cells. Furthermore, at all suboptimal densities of B7-1 examined, $CD8^+$ T cells required about twofold more B7-1 to achieve a similar level of activation as $CD4^+$ T cells.

If the density of B7-1 on the artificial APC rather than the absolute amount of B7-1 present in cultures is the limiting factor in co-stimulation, then the level of proliferation observed in cultures with microscpheres having a high density of B7-1 should not be achievable by adding higher numbers of microspheres with suboptimal levels of B7-1 to cultures. F23.1 was co-immobilized on microspheres with a high density of B7-1 or with B7-1 at an 11-fold lower level, and different numbers of these microspheres were co-cultured with responders (Fig. 5C). It is apparent from this figure that the lack of proliferation seen in cultures stimulated with F23.1 alone cannot be overcome by increasing the number of these microspheres added to cultures. More importantly, however, it is evident that co-stimulation is dependent on B7-1 density and not the total amount of B7-1 present in cultures; in this respect, both subsets of T cells

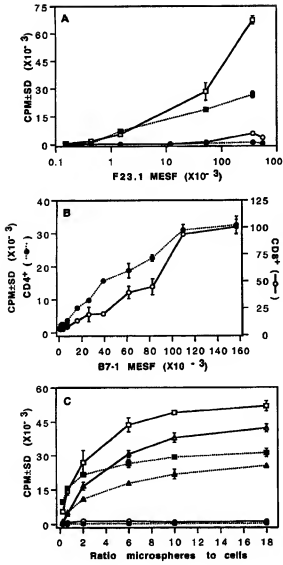

Figure 5. CD4⁺ and CD8⁺ T cells differ in sensitivity to B7-1 density. Results represent mean ± SD of triplicate cultures with 5×10^4 purified CD4⁺ (filled symbols, dashed line) or CD8⁺ (open symbols, solid line) T cells cultured for 48 h. (A) T cells were cultured with 5×10^5 microspheres bearing different densities of F23.1 with (squares) or without (circles) B7-1 co-immobilized. (B) T cells stimulated with 5×10^5 microspheres bearing a constant density of F23.1 and the indicated densities of B7-1 (MESF is a linear function of B7-1 density on microspheres). (C) T cells stimulated with different numbers of microspheres as indicated: F23.1-only microspheres, circles; F23.1/B7-1 microspheres bearing low B7-1 density, triangles; F23.1/B7-1 microspheres bearing high B7-1 density, squares.

demonstrated similar responses. Thus, co-stimulation is directly dependent on the density of B7-1 present on the ligand-presenting surface, and CD8⁺ T cells require higher levels of B7-1 to achieve levels of activation equivalent to those seen with CD4⁺ T cells.

3.5 Purified B7-1 does not co-stimulate *in trans*

In contrast to the co-stimulation observed when purified B7-1 is co-immobilized on the same surface as F23.1, no co-stimulatory activity was found when B7-1 was presented on a surface separate from, *i.e. trans* to, that providing the TCR stimulus. When F23.1 was immobilized on the surface of the culture plate and B7-1-bearing microspheres or fixed CHO-B7-1 were co-cultured with T cells, only the latter demonstrated co-stimulatory activity. Similar results were obtained over a wide range F23.1 and B7-1 sensities and input microsphere numbers (data not shown).

Table 1 summarizes the results of a representative mixing experiment in which responders were cultured with F23.1-bearing microspheres and with either B7-1-bearing microspheres, CHO-B7-1 cells, or LPS blasts. As expected based on work by others [5, 42, 43], both LPS blasts and CHO-B7-1 co-stimulated proliferation, although not as efficiently as purified B7-1 co-immobilized with F23.1 on the same surface. B7-1-bearing microspheres added *in trans*, however, did not co-stimulate proliferation in response to F23.1. This defect was not the result of inadequate B7-1 density on microspheres because quantitative flow cytometry demonstrated that B7-1-bearing microspheres had levels of B7-1 similar to CHO-B7-1 and microspheres bearing tenfold lower densities of B7-1 on the same surface as F23.1 efficiently co-stimulated proliferation. To rule out the possibility of inadequate adhesion between cells and B7-1-bearing microspheres, responders were cultured with F23.1-bearing microsphercs, and microspheres bearing B7-1 co-immobilized with ICAM-1 or the mAb Y3, which binds to MHC class I on T cell responders. Again, no co-stimulation was observed despite the presence of increased numbers of microsphere-cell conjugates, as shown by microscopic examination of these cultures (data not shown).

3.6 B7-1 co-stimulation results in distinct morphological changes in T cells

When cultures containing microspheres and cells are viewed using phase-contrast microscopy, microspheres appear dark in comparison to viable cells; thus, responding cells can be easily differentiated from stimulators (Fig. 6). Approximately equivalent numbers of microsphere-cell conjugates are apparent 12 h after the initiation of cultures containing F23.1 or F23.1/B7-1-bearing microspheres (Fig. 6A and B). At this time cells appear the same as at the initiation of culture and there is no significant cell/cell interaction observed. Isolated microsphere-cell conjugates and occasional small aggregates of microspheres and cells are apparent 36 h after the initiation of culture with F23.1-bearing microspheres (Fig. 6D). In cultures with F23.1/B7.1-bearing microspheres at this time point, numerous large aggregates are evident (Fig. 6E). Also, the latter cultures contain many elongated motile cells that are largely absent in cultures with microspheres bearing only F23.1. These motile cells can be seen associated with homotypic aggregates as well as individually, either alone or interacting with microspheres. These findings suggest that in addition to co-stimulating proliferation, B7-1 is capable of altering the phenotype of responding cells with respect to adhesion and motility.

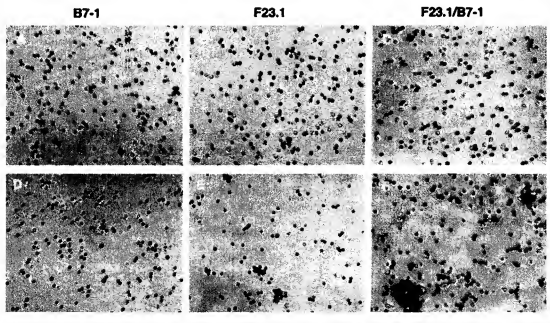

Figure 6. Microscopic analysis demonstrates distinct morphological changes associated with B7-1-mediated co-stimulation of T cell responses. Microspheres are 5 μm in diameter and appear dark in relation to cells. Nonadherent lymph node responders (5×10^4 per well) were cultured with 10^5 microspheres as indicated and photographed at 12 h (A, B and C) or 36 h (D, E and F). (A and D) B7-1 microspheres; (B and E) F23.1 microspheres; (C and F) F23.1/B7-1 microspheres. Essentially the same results were observed in purified $CD4^+$ and $CD8^+$ cultures (not shown).

Table 1. Purified B7-1 does not co-stimulate *in trans*

		cpm ± SD	
Stimulus[a)]	B7-1 MESF[b)]	$CD4^+$	$CD8^+$
Trans: F23.1 microspheres +			
Medium	–	187 ± 6	1079 ± 684
BSA microspheres	–	152 ± 15	716 ± 218
B7-1 microspheres	444217	154 ± 33	604 ± 27
CHO-B7-1	528327	8095 ± 912	22027 ± 1021
LPS blasts	2195	3577 ± 530	7415 ± 1159
Cis: F23.1/B7-1 microspheres			
High B7-1	493086	21650 ± 849	36253 ± 7073
Low B7-1	45513	10991 ± 1043	22511 ± 2297

a) $CD4^+$ or $CD8^+$ T cells (5×10^4) were cultured as described in Sect. 2.5 with 10^5 F23.1-coated microspheres and 10^5 of the indicated microspheres, 10^4 fixed CHO-B7-1 or 10^5 LPS blasts (*trans*); or with 10^5 microspheres having co-immobilized F23.1 and B7-1 (*cis*).

b) B7-1 MESF was calculated as described in Sect. 2.3, and is a linear function of the amount of B7-1 present on the indicated stimulator as determined by specific staining with the anti-B7-1 mAb 1G10.

4 Discussion

Differences in the response of $CD4^+$ and $CD8^+$ T cells have been noted in various studies, but in most cases, the APC required to activate $CD4^+$ versus $CD8^+$ cells differ. As a result, it is difficult to know whether differences in the responses observed are inherent to the two T cell subsets or whether they reflect differences in the stimulatory potential of the APC. The system used here, purified B7-1 co-immobilized on microspheres with anti-TCR mAb, allows a direct comparison of $CD4^+$ and $CD8^+$ responses to an identical stimulus. The results show that the dependence on B7-1 for co-stimulation is similar in some respects for both subsets. At the same time, there are also distinct differences that have implications for the induction of helper-independent CTL responses, and for the role of $CD4^+$ helper T cells in sustaining CTL responses.

Neither $CD8^+$ nor $CD4^+$ cultures responded significantly to microspheres bearing F23.1 alone at any density or microsphere number (Fig. 5). In immobilizing purified B7-1 on microspheres with anti-TCR mAb, it is shown that B7-1 is likely to be sufficient for the co-stimulation of TCR-mediated activation of both subsets of T cells. First, it is extremely unlikely that additional receptor/ligand interactions occur between the responding T cell and TCR ligand-presenting surface. Second, although the presence of co-stimulatory factors provided *in trans* by cells in cul-

ture cannot be ruled out, the use of T cells largely free of contaminating professional APC, the finding that mAb against B7-2 and ICAM-1 have no effect on the responses (Fig. 2), and the morphological observations showing that B7-1-dependent activation precedes significant cell/cell interaction (Fig. 6) all suggest that this is not likely to be the case.

In contrast to the results of Abe et al. [20] who found that CD28 cross-linking with mAb resulted in the preferential activation of $CD4^+$ T cells, results presented here, in accord with results reported by others [15, 16, 25], show that B7-1 does co-stimulated $CD8^+$ T cell responses. Also noteworthy with respect to $CD8^+$ responses is the lack of co-stimulation provided by the CD8 ligand K^b (Fig. 2). Work by others has shown that CD8 ligation by mAb results in the co-stimulation of proliferative responses when given in the context of suboptimal TCR stimulation [44]. One explanation for these differences may be related to the use of mAb to ligate CD28 or CD8 in those experiments. Alternatively, in the latter study, is the possibility that early after activation of resting $CD8^+$ cells, CD8 adhesion to K^b is insufficient to mediate signaling associated with co-stimulation of prolifertive responses.

Numerous studies indicate that although cell-associated B7-1 is capable of co-stimulating T cell activation *in trans* to the TCR stimulus, this co-stimulation is less efficient than that seen when the TCR ligand and B7-1 are present on the same cell [42, 43, 45]. Data presented in Table 1 indicate that levels of purified B7-1 equivalent to those present on CHO-B7-1 cannot co-stimulate $CD4^+$ or $CD8^+$ responses *in trans*, and since CHO-B7 are considerably larger in diameter then the 5-μm microspheres used in these studies, the actual density of B7-1 on their surface is likely to be at least fivefold higher than that present on CHO-B7. The ability of B7-1-expressing cells to provide co-stimulation *in trans* may be the result of the presence on cells of undefined T cell ligands that enable signaling through CD28 to occur in the absence of simultaneous TCR activation. Alternatively, the differences seen may be the result of steric factors associated with B7-1 presentation on rigid microspheres, as opposed to in the plasma membrane where the B7-1 is likely to be mobile. Regardless of the reason, this report shows that when separated from the complex milieu of cell surface proteins, the natural CD28 ligand B7-1 does not co-stimulate T cell proliferation *in trans* to the TCR stimulus.

The ease of distinguishing microspheres from responding cells facilitates examination of the morphology of responding cells in culture. Development of homotypic aggregation and motile forms beginning at 24 h after stimulation was routinely observed when B7-1 was present to provide a co-stimulatory signal (Fig. 6). The aggregation noted is suggestive of active T cell motility and such motility is observed in cultures (M. Deeths, unpublished observations). The relative absence of these phenomena in cultures stimulated with microspheres bearing F23.1 alone suggests that T cell contact with microspheres bearing co-stimulatory B7-1 may directly cause these changes. Thus, initial B7-1-dependent activation is likely to be independent of cell/cell contact. The formation of these aggregates also suggests B7-1-dependent alterations in the adhesive properties of the responding cell. No readily apparent differences in morphology are apparent upon microscopic examination of $CD4^+$ and $CD8^+$ cultures (M. Deeths, unpublished observations.) The B7-1-dependent changes in motility and adhesion are likely to have a profound impact on migration and effector function *in vivo*, and are in accord with studies demonstrating the up-regulation of integrin-mediated adhesion upon cross-linking of CD28 in resting human T cells [46, 47].

Despite the common requirement for B7-1 co-stimulation of proliferation and development of effector function in both $CD4^+$ and $CD8^+$ T cells, the responses to B7-1-mediated co-stimulation differ dramatically in the two T cell subsets. Although B7-1-mediated co-stimulation is required for the induction of IL-2 production in both T cell subsets, Fig. 3 shows that detectable IL-2 production is much lower and less prolonged in the $CD8^+$ subset. These differences may be the result of the terminal differentiation of pCTL into CTL that have lost the ability to make IL-2, higher CTLA-4 expression on responding $CD8^+$ T cells leading to more effective down-regulation of IL-2 production in this subset, or both [27, 28]. Ultimately, the qualitative differences observed are likely to be the manifestation of differential developmental programs in the two subsets of naive cells. Thus, in $CD4^+$ helper T cell precursors, B7-1 co-stimulation results in efficient cytokine production (Figs. 2 and 3), while in the $CD8^+$ pCTL subset, B7-1 co-stimulation results in limited IL-2 production, rapid clonal expansion, and development of cytolytic activity (Figs. 3 and 4).

It is apparent from Fig. 3. that the limited IL-2 production seen in $CD8^+$ cultures correlates with loss of cell viability and an abrupt decline in proliferation. In experiments performed by Sprent and Schaefer [48], similar results were noted using purified $CD8^+$ and $CD4^+$ T cells in class I or II-restricted MLR, and it was suggested that the basis for the fall in proliferation in $CD8^+$ cultures after day 3 was the result of the development of CTL activity and the destruction of stimulators. However, the use here of an artificial ligand-bearing surface helps to ensure that the effects observed are not the result of effects produced on the APC by the different effector functions induced in the two T cell subsets, such as cytotoxic activity. On day 5 of culture, microspheres analyzed by flow cytometry showed levels of B7-1 and F23.1 less than twofold different from those found before culture (data not shown). Thus, it appears that B7-1 co-stimulation allows only the induction of a helper-independent CTL response, but cannot sustain it despite the continued presence of the stimulatory ligands. *In vivo*, numerous alternate co-stimulatory signals may act to sustain initially helper-independent/B7-1-dependent CTL responses. Not mutually exclusive is the possibility that prolonged CTL responses, regardless of the initiating stimulus, may be inherently helper-dependent. In this regard, it is noteworthy that on day 4, when IL-2 production by $CD8^+$ T cells is nearly undetectable, $CD4^+$ IL-2 production is maximal (Fig. 3). In experiments not shown here, the addition of exogenous cytokines to B7-1 co-stimulated $CD8^+$ cultures on day 3 resulted in prolonged proliferation and cell survival.

The dependence of co-stimulation on B7-1 density present on the surface presenting the TCR stimulus in the range shown in Fig. 5B encompasses the levels of B7-1 found on

professional APC (Table 1 and data not shown). The finding that at suboptimal B7-1 densities, $CD8^+$ T cells require higher densities of B7-1 to reach a relative state of activation equivalent to that seen with $CD4^+$ cells, could be the result of lower CD28 expression on resting $CD8^+$ T cells [8], higher expression of the inhibitory B7-1 receptor CTLA4 on this subset following activation, or both [27, 28]. Gajewski [25] has shown that B7-1, but not B7-2, efficiently co-stimulates $CD8^+$ T cell activation, and the results were interpreted in the context of the differential kinetics of B7-1 and B7-2 binding to CTLA4 and CD28. That is, if $CD8^+$ T cells (relative to $CD4^+$ T cells) had a higher threshold requirement for CD28 ligand expression on APC, then B7-1, with a slower off-rate and higher affinity for CD28 and CTLA4 than B7-2 [23], would be a better co-stimulatory ligand for these cells. The data presented here (Fig. 5B) support the hypothesis that $CD8^+$ T cells have a higher requirement for B7-mediated co-stimulation.

Work by Abe et al. [20] has shown that B cell-enriched APC provide poor co-stimulation to anti-CD3-treated $CD8^+$ T cells, while this population of accessory cells very efficiently co-stimulates $CD4^+$ responses to the same anti-CD3 stimulus. Also, it has been found that upon activation, B7-2 is expressed earlier and at higher levels on B cell blasts than is B7-1 [22, 37, 49] and that the predominant co-stimulatory function of B cells early after activation is provided by B7-2 rather than B7-1 [37]. Finally, work by others has demonstrated the uptake and presentation of exogenous Ag in the class I pathway by macrophages *in vitro* [50–52], and recent results *in vivo* suggest that a macrophage or macrophage-like host cell is responsible for the phenomenon of cross-priming to tumor Ag in the context of self-MHC class I [53]. Since, unlike B cells, macrophages may express at least as much functional B7-1 as B7-2 [22, 29], a reasonable hypothesis is that macrophages (which would be activated by $CD8^+$ T cell-derived IFN-γ), are better suited to activate CTL responses to phagocytosed tumor and viral particulates, while B cells (which require helper derived cytokines for full activation) are more suited to activate T helper responses after Ag uptake via the B cell antigen receptor. Given these findings, and the difficulty in comparing results obtained with purified or cell-associated ligands, the purification of mouse B7-2 is presently underway in this laboratory.

In conclusion, the results presented here show that B7-1 provides a co-stimulatory signal that allows the efficient activation of both $CD4^+$ and $CD8^+$ T cells as shown by the induction of effector function in both T cell subsets, as well as by the development of morphological and adhesive changes likely to be important in the expression of this effector function. While $CD4^+$ responses were sustained, $CD8^+$ responses were transient, suggesting that for $CD8^+$ T cells, the maintenance of effector function as measured by the presence in cultures of helper-independent CTL activity requires co-stimulatory ligands distinct from B7-1. Alternatively prolonged CTL responses may be inherently dependent on exogenous cytokines derived either from helper T cells or other cell types present *in vivo*. The data also show that $CD8^+$ T cells are less responsive to suboptimal densities of B7-1 on the ligand-bearing surface than are $CD4^+$ T cells, and thus require higher densities of B7-1 to achieve similar levels of activation, suggesting that the level of B7-1 expression the APC may influence the type of T cell response induced by that APC.

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EXHIBIT 6

Response Differences between Human CD4$^+$ and CD8$^+$ T-Cells during CD28 Costimulation: Implications for Immune Cell-Based Therapies and Studies Related to the Expansion of Double-Positive T-Cells during Aging

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Since CD28 costimulation is critical for T-cell activation, there is great interest in CD28 as a target for immuntherapeutic approaches. We show that stimulation of human CD4$^+$ and CD8$^+$ T-cells differs in their responsiveness to stimulation with anti-CD3/CD28-coated beads, as surrogate antigen-presenting cells. While the CD4$^+$ subset responded with sustained proliferation, CD8$^+$ T-cells grew for a limited period only and failed to produce IL-2 beyond the first few days in culture. This decrease is accompanied with an increased rate of apoptosis in CD8$^+$ T-cells despite Bcl-x_L expression. The CD8$^-$ but not the CD4$^+$ subset developed a reversible double-positive phenotype during CD28 costimulation. This finding may have some bearing on the appearance of double-positive T-cells in human peripheral blood. This double-positive subset was shown to undergo a statistically significantly increase during aging in humans. Taken together, the above data have important implications for immunotherapy and immune senescence. © 2000 Academic Press

Key Words: CD28 costimulation; T-lymphocytes; T-cell subsets; double-positive PBL; aging; apoptosis.

INTRODUCTION

The T-cell antigen receptor (TCR) and the CD28 accessory receptor synergize in the activation of T-lymphocytes, including stimulation of cellular proliferation, prevention of apoptosis, and maintenance of antigen responsiveness (1–3). According to current immunological dogma, this receptor combination is responsible for the integrated delivery of two signals, also known as signals 1 and 2, which induce IL-2 transcription, cytokine message stabilization, Bcl-x_L expression, and suppression of anergy-inducing factors (3–8). However, since most of the support for the delivery of a second signal has been generated in CD4$^+$ T-cells, much less is known about the biological effects of CD28 costimulation on human CD8$^+$ T-cells (9). Indeed, it is clear from studies on murine T-lymphocytes that there are differences in the response of the CD4$^+$ and CD8$^+$ subsets during CD28 costimulation (10–12). While, for instance, murine CD4$^+$ T-cells mount a vigorous and sustained proliferative response to TCR/CD28 coligation, the CD8$^+$ subset only proliferates for a limited time period (10, 12). Murine CD4$^+$ and CD8$^+$ T-cell subsets also differ with respect to the effect of CD28 on anergy induction (12). While CD28 costimulation prevents antigen nonresponsiveness in CD4$^+$ cells, CD8$^+$ T-cells can be anergized even in the presence of CD28 costimulation (12).

Despite the substantial interest in CD28 as a target for modifying immune reactivity in humans (13), very little is known about the role of this receptor in CD8$^+$ T-cells. Because of the importance of cytotoxic T-cells in such conditions as cancer and autoimmunity, it is critical to learn more about the role of CD28 in CD8$^+$ T-cells in humans. The extent to which this type of information can be exploited is exemplified by the use of anti-CD3 + CD28 conjugated magnetic beads to expand CD4$^+$ T-cells *ex vivo* (14). These cells can be used for adoptive immunotherapy in conditions like HIV-1 infection (15, 16). Not only it is comparatively difficult to expand CD8$^+$ T-cells in similar circumstances, but also these cells have a reduced survival rate in cell culture. Moreover, our preliminary data showed the propensity of the CD8$^+$, but not the CD4$^+$ subset to develop a double-positive phenotype (CD4$^+$/CD8$^+$) during CD28 costimulation. While expression of the CD4 receptor on CD8$^+$ T-lymphocytes is of possible pathophysiological significance, e.g., rendering them susceptible to HIV infection (17, 18), the physiological significance of the DP phenotype in healthy individuals is unclear. It has been shown, however, that human peripheral blood contains a small percentage (1–2%) of

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double-positive (DP) T-cells (19–21) and that, in non-human primates which express a larger pool of DP T-cells in the peripheral blood, there is a progressive rise in this subset with aging (22–26).

We wished to determine whether the human $CD4^+$ and $CD8^+$ T-cell subsets differ in their growth and survival characteristics during stimulation with a surrogate antigen-presenting cell, i.e., anti-CD3 and anti-CD28 mAb conjugated to microspheres (14). In addition, we sought to determine whether the pool of double-positive T-cells is expanded during aging in humans. We show here that while the human $CD8^+$ subset is responsive to CD28 costimulation, these cells abort their proliferative response in parallel with a failure to produce IL-2 after 3 days in culture. While there was a higher rate of apoptosis in $CD8^+$ vs $CD4^+$ T-cells, these subsets did not differ in the expression of the anti-apoptotic protein, Bcl-x_L. Although the acquisition of a DP phenotype on the $CD8^+$ subset is reversible *in vitro*, the percentage of DP T-cells in human peripheral blood increases with age. These findings have important implications for studies using $CD8^+$ lymphocytes for adoptive immunotherapy, as well as functional changes in the immune system during aging.

MATERIALS AND METHODS

Reagents

The anti-CD3 mAb (OKT3) was acquired from Ortho Pharmaceuticals (Raritan, NJ) and the CD28 mAb (9.3) was provided by Bristol–Myers Squibb (Seattle, WA). CD80 (B7-1) and CD86 (B7-2) IgG fusion proteins were produced as described (27). The anti-Fas mAb (CH 11) was purchased from MBL (Nagoya, Japan). The FITC-labeled anti-CD8 mAb (Leu-2a) and the PE-labeled anti-CD4 mAb (Leu-3a) were from Becton–Dickinson (Montain View, CA). Tosyl-activated magnetic beads M-450 and the CD4 and CD8 isolation kits were purchased from Dynal (Lake Success, NY). Anti-Bcl-x_L antibodies were obtained from PharMingen (San Diego, CA). The annexin V–FITC kit was purchased from Trevigen (Gaithersburg, MD).

Coupling of OKT3 and 9.3 mAb to Tosyl-Activated Dynal Beads

OKT3 and 9.3 mAb were bound to tosyl-activated magnetic beads M-450 (Dynal) as previously described (14). Briefly, 4×10^8 beads were incubated with 150 μg mAb at a ratio of 1:1, 1:9, or 9:1 in 1 ml of 0.1 M borate buffer for 24 h at 37°C. After coupling, beads were resuspended to 4×10^7 beads/ml and 360 μl of this solution was used to stimulate 5×10^6 target cells.

Preparation and in Vitro Culture of Primary $CD4^+$ and $CD8^+$ T-Cells

PBMCs were isolated from human peripheral blood of healthy individuals by Ficoll Hypaque density gradient centrifugation (IsoPrep, Robbins Scientific, Sunnyvale, CA). $CD4^+$ and $CD8^+$ T-cells were generally purified by positive selection using anti-CD4 and anti-CD8 Dynabeads according to the manufacturer's instructions (Dynal) (28). In the experiments performed in Fig. 1, the $CD28^+CD4^+$ T-cells were purified by a negative selection method using magnetic beads (Dynal) as described previously (14). Flow cytometry confirmed the percentage of enhancement of $CD4^+$ and $CD8^+$ T-cells as >98% by either positive or negative enrichment procedures. The purified T-cells were cultured at 1×10^6 cells/ml in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (pH 7.4) and stimulated with 4 to 10 anti-CD3/anti-CD28-coated beads per cell in the absence of feeder cells (14, 27). The cultures were fed at 2- to 3-day intervals. Cells were detached from the antibody-coated beads by pipetting until the clumps of cells had been dispersed and fresh medium was added so that the cells were maintained at a concentration of 2×10^6 cells/ml. Jurkat T-cells were grown in the same medium described above.

IL-2 Measurement

Purified $CD4^+$ and $CD8^+$ T-cells at a concentration of 10^6 cells/ml were stimulated with anti-CD3/anti-CD28-coated beads in tissue culture dishes. To determine lymphokine production, small aliquots of culture supernatant were harvested at the indicated time points and analyzed by ELISA for IL-2 production (UMAB Cytokine Core Laboratory, Baltimore, MD).

Human Subjects and Statistical Methods

Heparinized blood was obtained from 9 healthy young donors with mean age 29 ± 8 years and 29 elderly donors, mean age 87 ± 6.5 years. This blood was used for CD4/CD8 phenotyping. The results were analyzed by Student's *t*-test, using Microsoft Exel 97.

Phenotype Analysis

Phenotype analysis for bead-activated lymphocytes was done by direct immunostaining. Stimulated $CD4^+$ and $CD8^+$ T-cells were removed from the beads and washed in PBS. A total of 5×10^5 cells were resuspended in 100 μl of PBS and stained with 5 μl of FITC-labeled anti-CD8 mAb and 5 μl of PE-labeled anti-CD4 mAb. After 30 min of incubation on ice, cells

were washed three times with PBS and analyzed by flow cytometry. Phenotyping of peripheral blood lymphocytes was done by adding 5 μl of the same antibody reagents to 50 ml of whole blood in 12 × 75 mm culture flasks. After incubation in the dark at room temperature for 20 min, erythrocytes were lysed twice with 500 μl of FACS lysis buffer during a 2-min incubation period. The cells were washed twice with PBS containing 0.1% sodium azide, resuspended in PBS, and analyzed by flow cytometry.

Annexin V/PI Staining

Stimulated $CD4^+$ and $CD8^+$ T-cells were removed from the beads and washed in PBS. A total of 5×10^5 cells were resuspended in annexin V/PI staining buffer according to the manufacturer's instructions (29). After incubation at room temperature for 15 min, samples were analyzed by flow cytometry.

Flow Cytometry

Flow cytometric analyses were performed using a FACScan (Becton–Dickinson) equipped with a single 488-nm argon laser. Forward and side scatter analysis was used to gate out dead cell fragments and 10,000 gated events were acquired and analyzed (29). FITC fluorescence was analyzed at the excitation and emission settings of 488 and 535 nm (FL-1 channel), respectively, while PI analysis was performed at the excitation and emission settings of 488 and 575 nm (FL-2 channel), respectively.

Western Blotting Analysis for Bcl-x_L

Cells were lysed in SDS buffer and 50 μg of cellular lysate was separated by 12% SDS–PAGE and transferred to PVDF membranes (28). Immunoblotting was performed using a 1:1000 dilution of the anti-Bcl-x_L mAb, followed by a 1:2000 dilution of HRP-coupled anti-mouse Ab and detection by ECL.

RESULTS

Human $CD4^+$ and $CD8^+$ T-Cell Subsets Differ in Proliferation and Survival Responses during Stimulation with Anti-CD3 + Anti-CD28

Clinical trials are currently being conducted in humans in which large-scale *ex vivo* expansion of $CD4^+$ T-cells is used to treat HIV-1-infected individuals in an attempt to correct their cellular deficiency (15, 16). Cellular expansion is achieved by culturing these cells with anti-CD3 (OKT3) plus anti-CD28 (9.3) mAb immobilized on microspheres (14). These microspheres act as surrogate APC, which induce polyclonal T-cell activation, and the beads can be removed by passage through a magnetic field before infusion of the activated lymphocytes (14–16). In the development of this type of immunotherapy, we have compared the proliferative responses of $CD4^+$ and $CD8^+$ subsets using different amounts of stimulatory anti-CD3 and anti-CD28 mAb (Fig. 1A). Beads were coated with 9:1, 1:1, and 1:9 molar ratios of anti-CD3 and anti-CD28 mAb. Previous experiments have shown that for human $CD4^+$ cells, a 1:1 ratio of these reagents is optimal for their long-term proliferation (data not shown). In a representative experiment on the T-cells of a healthy donor, we observed that at equimolar (1:1) antibody concentrations, the $CD4^+$ subset undergoes a vigorous and continued expansion for a 60-day time period (Fig. 1A). In contrast, $CD8^+$ lymphocytes had a markedly reduced growth potential in this culture system and became nonresponsive after 2 to 3 weeks of culture, as indicated by a plateau on the growth curve (Fig. 1A). These differences were even more accentuated when the antibody ratio was increased to 9:1 in favor of OKT3 (Fig. 1A). While $CD4^+$ proliferation was about the same as at a 1:1 antibody ratio, the net accumulation of $CD8^+$ T-cells ceased after 20 days of stimulation (Fig. 1A). The $CD8^+$ subset was nonresponsive when the antibody concentration was reversed to 1:9 in favor of the anti-CD28 mAb (data not shown).

The affinity of the anti-CD28 antibody (mAb 9.3) for CD28 is much higher than that of the natural B7 ligands (30). It is possible, therefore, that the difference in the growth potential between the $CD4^+$ and $CD8^+$ cells observed following ligation with antibody-coated beads was a consequence of the longer dwell time and slower off rate of the antibody. To test this possibility, a repeat of the same experiment was done using beads coated with the natural CD28 and CTLA-4 ligands, i.e., CD80 (B7-1) and CD86 (B7-2). Beads were prepared with fusion proteins coated with human CD80Ig or CD86Ig, and OKT3 at 1:1 molar ratios. The growth curves of cells stimulated with natural costimulatory ligands confirmed the response differences between the $CD4^+$ and $CD8^+$ T-cells. Thus, while CD86Ig induced a vigorous and sustained proliferative response in $CD4^+$ T-cells, the response of the $CD8^+$ T-cell subset was almost two orders of magnitude less (Fig. 1B). Moreover, the $CD8^+$ T-cell subset ceased to proliferate after days 10–18 (Fig. 1B). Interestingly, CD80Ig was also a poor costimulatory ligand for the $CD8^+$ T-cells (Fig. 1B). Previous studies have shown that CD86 is more effective than CD80 at inducing proliferation of human $CD4^+$ T-cells (27). Taken together, the data in Fig. 1 show that human peripheral blood $CD8^+$ T-cells did not have the same long-term proliferation as the $CD4^+$ subset after CD3/CD28 costimulation. Similar response differences have been demon-

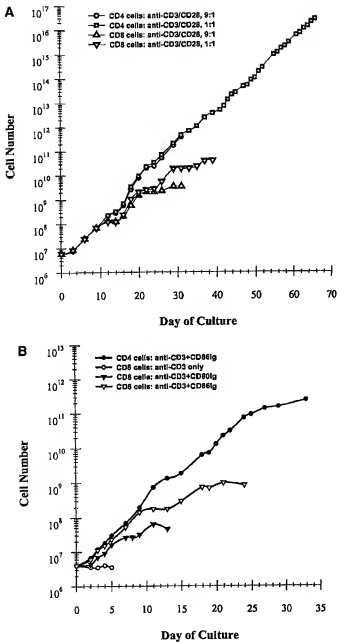


FIG. 1. CD4$^+$ and CD8$^+$ T-cells differ in their polyclonal expansion potential in response to CD3/CD28 or CD3/B7 costimulation. (A) Purified CD4$^+$ and CD8$^+$ T-cells were stimulated with magnetic beads bearing anti-CD3 and anti-CD28 mAb. (B) Purified CD4$^+$ and CD8$^+$ T-cells were stimulated with magnetic beads bearing anti-CD3 and CD80Ig or CD86Ig fusion proteins. Fresh medium was added to the cultures as required and excess cells were discarded. Cell number was determined using the average of two counts on a Coulter Counter ZM. The total number of cells that would be expected to accumulate is displayed, taking into account discarded cells. Electronic cell counting was confirmed by manual determination of viable cells by trypan blue exclusion and hemacytometer counting on the indicated days following stimulation. Fresh beads were added at 1- to 2-week intervals as previously described (14).

strated in murine CD4$^+$ and CD8$^+$ T-cell subsets obtained from the spleen (10).

The failure of the CD8$^+$ subset to expand as rapidly and as sustainably as the CD4$^+$ subset is accompanied by an increased rate of cell death in the former compared to the latter subset (Fig. 2A). While the time window during which this difference became noticable varied somewhat from donor to donor, it was generally obvious within the first 10 days of cellular stimulation. Using the blood from the same donor shown in Fig. 2A, two-color annexin V/PI staining showed an increase in the percentage of apoptotic cells in the CD8$^+$ (57%) compared to the CD4$^+$ (14%) subset by day 5 (Fig. 2B). While the rates and progress of apoptosis differed from donor to donor, they were always higher for the CD8$^+$ compared to the CD4$^+$ population. This suggests that the anti-apoptotic effects of the CD28 receptor may be lost or diminished in the CD8$^+$ subset. While it is known that CD8$^+$ lymphocytes lose CD28 expression after prolonged time periods of stimulation, flow cytometry assessment of CD28 expression did not show decreased staining intensity over the course of the stimulation periods used in this study. Since one of the major functional effects of CD28 is Bcl-x_L expression, we asked whether there are differences in the relative abundance of Bcl-x_L expression in CD8$^+$ and CD4$^+$ T-lymphocytes. Immunoblotting demonstrated, however, that both subsets express roughly equal amounts of Bcl-x_L during CD28 costimulation (Fig. 3A). While this suggests that Bcl-x_L expression is relatively inefficient in preventing anti-CD3 induced apoptosis in CD8$^+$ T-cells, it is interesting that exposure of the same subset to anti-Fas mAb or TNF-α could not enhance the rate of immunobead-induced cell death (Fig. 3B). This suggests that Bcl-x_L expression by CD28 costimulation may have a protective effect against these apoptotic stimuli. That is further substantiated by the inability of the same stimuli to induce apoptosis in CD4$^+$ T-cells (Fig. 3B). These findings are in agreement with previously demonstrated effects of Bcl-x_L on Fas and TNF-α-induced apoptosis (31–33). Interestingly, CD28 did not protect against staurosporine- or menadione-induced apoptosis in either T-cell subset (Fig. 3C). The former acts directly on mitochondria to release apoptogenic factors (34). Moreover, our use of a Jurkat cell line, which does not produce Bcl-x_L under basal or stimulatory conditions has a high rate of TNF-α and Fas-induced apoptosis (Fig. 3B).

Since Bcl-x_L expression failed to provide an explanation for why CD8$^+$ T-cells die faster than CD4$^+$ T-cells, we investigated the role of IL-2 production in the CD4$^+$ and CD8$^+$ subsets. IL-2 has important effects on T-cell survival (35) and is a key element in the cellular response to CD28 costimulation (4, 6). Assessment of IL-2 production in the supernatant of CD3/CD28-stimulated human T-cell subsets showed that, while CD4$^+$

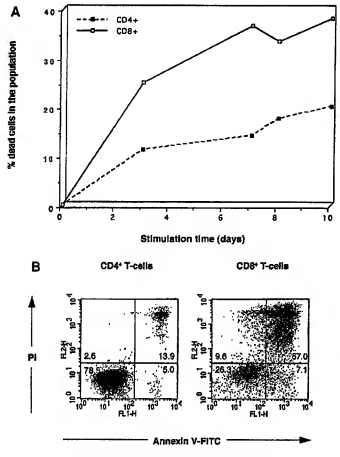


FIG. 2. CD4+ and CD8+ T-cells differ in their rate of cell death in response to CD3/CD28 costimulation. CD4+ and CD8+ T-cells were purified from PBMCs and stimulated with magnetic beads bearing anti-CD3 and anti-CD28. (A) Kinetics of cell death is shown by the number of dead cells in the population as determined by trypan blue exclusion and hemacytometer counting on the indicated days. (B) Two-color annexin V/PI analysis of CD4+ and CD8+ T-cell blasts of a representative donor 5 days after stimulation with anti-CD3/CD28 beads. The time period during which CD8+ T-cells began to show increased rates of cell death varied from days 5 to 10.

T-cells had sustained IL-2 accumulation over a 10-day observation period, CD8+ T-cells failed to produce this cytokine beyond the first few days in culture (Fig. 4A). Interestingly, the addition of endogenous IL-2 slowed the rate of apoptosis in the CD8+ subset. Figure 4B shows a representative experiment where CD8+ T-cells were grown in the absence and presence of rIL-2. Cells remained viable and growing for 7 days in both populations. Beyond day 7 the expanded CD8+ population grown in the absence of exogenous IL-2 showed increased rates of cell death while the rate of death remained stable in the rIL-2+ population. This death event included apoptotic cells as shown by the increased percentage of annexin V/PI-positive cells in the rIL-2− population on day 8 (Fig. 4B). This suggests that IL-2 withdrawal is an important stimulus for cell death in CD8+ T-cells.

The CD8+ but Not the CD4+ Subset Developed a Double-Positive Phenotype during CD28 Costimulation

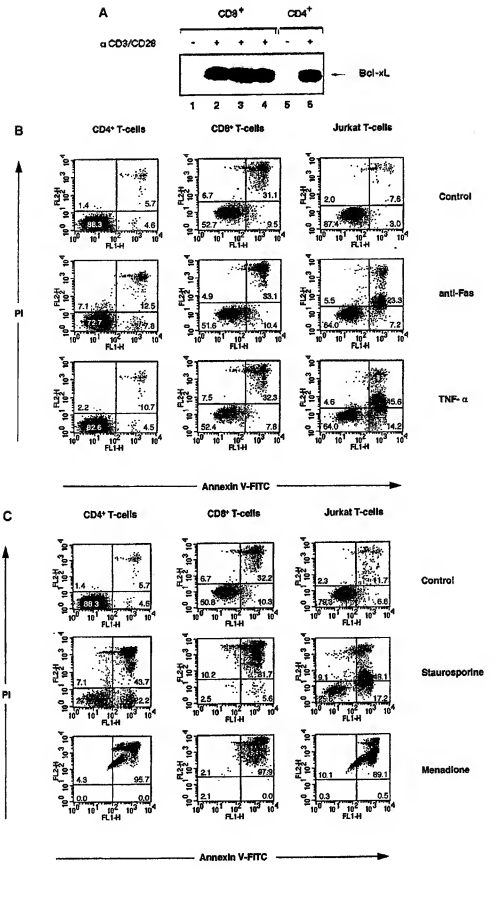
Based on data showing that CD8+ T-cells express the CD4 receptor and are rendered susceptible to HIV-1 infection during CD28 costimulation (17, 18), we followed the rate of CD4/CD8 expression on CD4+ and CD8+ subsets during stimulation with OKT3/9.3-conjugated beads. While the CD4+ subset remained >99% CD4+ and expressed the CD8 receptor on <1% of the cells over a 21-day observation period, purified CD8+ T-cells showed a high rate of conversion to a double-positive status. A representative experiment shown in Fig. 5 shows that the percentage of dual-positive cells in the CD8+ subset reached >80% by day 7 and then decreased to 44 and 2.5% by days 11 and 21, respectively (Fig. 5). This suggests that although the development of a DP phenotype is reversible, it is the CD8+ rather than the CD4+ subset which gives rise to the appearance of DP T-cells in the peripheral immune system.

Expansion of the DP Phenotype in Humans during Aging

While the physiological significance of CD4 expression on CD8+ T-cells is unknown, human peripheral blood contains between 1 and 2% DP T-cells (19–21). Although there are several reports showing an expanded DP subset in disease, it has been demonstrated that the presence of a large number of DP cells in humans is compatible with normal immune function (36–40). Moreover, it has been shown that in animals such as cynomolgus monkeys and swine, there is a progressive rise in the percentage of DP T-cells in the blood during aging (25, 41, 42). We asked, therefore, whether similar changes occur in humans and assessed the percentage of DP T-cells in the blood of young and older adults. This analysis yielded a statistically significant ($P < 0.002$) increase in the percentage of DP T-cells in older vs younger adults (Fig. 6). Thus, while the mean value in the older age group amounted to 2.6% (variance 5.3%), the corresponding value in younger adults was 0.9% (variance 0.33%).

DISCUSSION

The activation requirements of functionally specialized T-cell subsets have been an area of intensive investigation. Not only is this knowledge helpful in understanding differences in T-cell function, e.g., helper


A
CD8+
CD4+
α CD3/CD28
- + + + - +
Bcl-xL
1 2 3 4 5 6
B
CD4+ T-cells
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Jurkat T-cells
Control
anti-Fas
TNF-α
PI
Annexin V-FITC
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and cytotoxic abilities, but also it presents an opportunity to regulate cellular immune function in disease. The role of the CD28 receptor is key in understanding these differences due to its influence on cellular activation threshold, cytokine production, mRNA ability, and the prevention of apoptosis and anergy (3–8). According to a widely accepted hypothesis regarding T-cell activation, CD28 delivers one of the two major signals which are required for T-cell responsiveness (7, 8). While the validity of this two-signal model has been substantiated in the CD4⁺ T-cell subset, it is becoming increasingly clear that this model may not be applicable to both T-cell subsets (9, 43, 44). Among the variables that need to be considered is the affinity of the TCR for its ligand and other coaccessory receptors. Several groups have now shown that certain T-cells can respond to signal 1 alone, provided that the affinity of TCR for antigen is sufficiently strong (9, 43, 44). Moreover, antigen presentation to CD8⁺ T-cells is often done by nonprofessional APC, which lack the ligands (CD80/CD86) which are required for delivery of signal 2 (45).

The response differences of CD4⁺ and CD8⁺ lymphocytes to ligation of the CD28 receptor are highly relevant to attempts at modifying T-cell function in patients with autoimmune disease, asthma, cancer, and infectious diseases (13). In addition to the use of interfering ligands for this receptor, e.g., CTLA-4 recombinant protein or mono- and bifunctional antibody reagents (13), there are also clinical trials which attempt to modify immune function by reinfusion of *ex vivo* expanded T-cells (14–16). One example is treating HIV-1-infected patients with a CD4⁺ T-cell subset which is expanded *ex vivo* by anti-CD3 plus anti-CD28 mAb conjugated to microspheres (14–16). This approach is feasible because the CD4⁺ subset readily responds to those surrogate APC with proliferation (16). Should a similar approach be desired for the CD8⁺ subset, e.g., *ex vivo* expansion of tumor-infiltrating lymphocytes, this protocol will need to be modified because of the poor proliferative response of CD8⁺ T-cells to CD28 costimulation. Based on studies in murine T-lymphocytes, this goal may be accomplished through the use of IL-6 or TNF-α, which synergize with anti-CD3 mAb *in vitro* (9).

Although the exact reason for the poor proliferative response of human CD8⁺ T-cells is not properly understood, we did observe that these cells have a limited

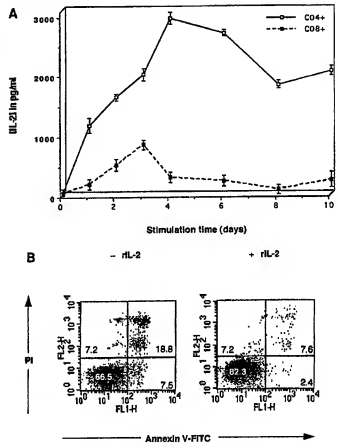

FIG. 4. Limited IL-2 production in CD8⁺ T-cells correlates with a higher rate of cell death. (A) ELISA assay showing IL-2 concentration in the culture medium of the same donor shown in Fig. 2. CD4⁺ and CD8⁺ T-cells were purified from PBMCs and grown in the presence of anti-CD3/CD28-conjugated beads as described in Fig. 1. Results represent means ± SD of triplicate measurements. (B) Two-color annexin V/PI analysis of CD8⁺ T-cell blasts from a different donor on day 8 after introduction of anti-CD3/CD28 beads in the absence and presence of 50 ng/ml rIL-2. Over the same time period, the population grown in the presence of rIL-2 expanded 75-fold, while the cells grown in the absence of rIL-2 only multiplied 20-fold. The rate of cell death accelerated 2.5-fold in the rIL-2-negative population over the subsequent 24 h.

capacity to produce IL-2 in culture (Fig. 4). While CD8⁺ cultures produce IL-2 for *a few* days only, CD4⁺ T-cells maintained cytokine production for the entire observation period (Fig. 4). Similar observations have been made for murine T-cell subsets, where the presence of

FIG. 3. Bcl-x_L expression does not prevent apoptosis in CD8⁺ T-cell blasts. (A) Western blot analysis of Bcl-x_L in CD4⁺ and CD8⁺ T-cells after stimulation with anti-CD3/CD28-conjugated beads. Lanes 1 and 5 represent unstimulated cells, while lanes 2–4 show Bcl-x_L expression in CD8⁺ T-cell blasts at days 5, 6, and 7 and lane 6 in CD4⁺ T-cells at day 5. (B) Two-color annexin V/PI staining showing the effect of anti-Fas (100 ng/ml) and TNFα (50 ng/ml) + cyclohexamide (10 mM) treatment of T-cells blasts. Cells were exposed for 16 h. The CD4⁺ and CD8⁺ blasts were obtained by removal of beads on day 6. Numbers in each quadrant represent percentage of cells. (C) A similar analysis to show the effect of 1 μM staurosporine and 25 μM menadione treatment for 16 h.

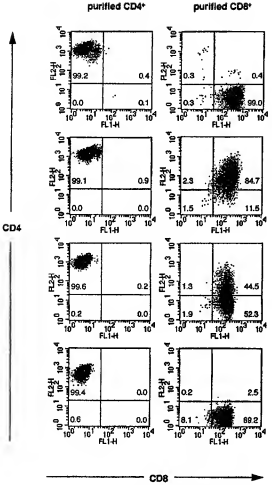

FIG. 5. $CD8^+$ T-cells become double positive in response to CD3/CD28 costimulation. Two-color flow cytometric analysis of CD4 and CD8 expression on purified $CD4^+$ and $CD8^+$ T-cells after stimulation with anti-CD3/CD28-conjugated beads. Cells were removed from the beads at the indicated time points and stained for CD4 and CD8 expression. Numbers represent the percentage of positive cells in each quadrant. Purity of the $CD4^+$ and $CD8^+$ depletion is represented by the staining profile for day 0 (unstimulated cells).

$CD4^+$ T-cells is able to support a sustained $CD8^+$ T-cell response (45, 46). Although CD28 costimulation contributes to the activation of the NF-kB and Jun kinase cascades which are required for optimal activation of the IL-2 promoter (28, 47), there is no evidence to suggest that these signaling cascades are deficient in murine $CD8^+$ T-cells. An alternative explanation may be in the processing of anergic signals in these subsets. In this regard, CD28 is known to suppress the induction of anergy factors in T-cells (8, 9). A supposed target for these negative regulatory factors is the IL-2 promoter (8, 9). One possibility is that the CD28 receptor fails to suppress anergy factors in $CD8^+$ T-cells. This notion is consistent with the observation that CD28 costimulation does not prevent anergy induction in murine $CD8^+$ T-cells (12). This aspect is important for the intended use of CD28-costimulated $CD8^+$ T-cells for adoptive immunotherapy, as one would like to avoid the administration of anergized T-cells.

CD28-costimulated $CD8^+$ T-cells exhibit a high rate of apoptosis beyond the third day of culture (Fig. 2B). This represents another difference from $CD4^+$ T-lymphocytes, in which CD28 exerts potent anti-apoptotic effects. A key contributor to the anti-apoptotic effects of CD28 is Bcl-x_L (2). However, this protein was equally expressed in $CD4^+$ and $CD8^+$ lymphocytes and can therefore not explain the higher apoptotic rate in $CD8^+$ T-cells (Fig. 3A). Instead, IL-2 withdrawal may constitute the principal mechanism leading to apoptosis in $CD8^+$ T-cells since replenishment of cultures with IL-2 decreased the rate of cell death (Fig. 4). That does not exclude an anti-apoptotic role for Bcl-x_L in $CD8^+$ T-cells, because treatment of the latter subset with anti-Fas or TNF-α did not further increase the rate of cell death (Fig. 3B). While this notion remains to be proven in $CD8^+$ T-cells, it has been demonstrated previously that Bcl-x_L protects against anti-Fas- and TNF-α-induced apoptosis (2, 31–33). Moreover, Jurkat T-cells which do not express Bcl-x_L under basal or stimulated conditions have a high rate of apoptosis under similar stimulatory conditions (Fig. 3B). Although Bcl-x_L exerts its anti-apoptotic effects in the outer mitochondrial membrane (32), it is interesting that staurosporine, which targets the mitochondrial membrane, was an effective inducer of apoptosis in CD28 costimulated cells (Fig. 3C). Similarly, Bcl-x_L expression failed to protect against menadione-induced apoptosis (Fig. 3C). A possible contribution by the CD4 receptor to apoptosis prevention also needs to be considered in the $CD8^+$

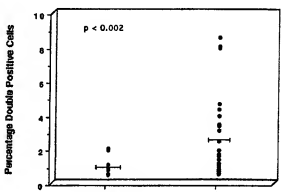

FIG. 6. Scatter plot to show the increase in the percentage of DP lymphocytes in younger vs older human subjects. PBL freshly isolated from peripheral blood of young (mean age 29 ± 8 years, $n = 9$) and old donors (mean age 8 ± 6.5 years, $n = 29$) were stained for CD4 and CD8 and analyzed by flow cytometry. Each point represents the value corresponding to one subject and the horizontal bar indicates the mean for each set.

subset, because the majority of $CD8^+$ T-cells which survive beyond 3 days are DP (Fig. 5). Such an effect may be explained by the ability of CD4 to modify TCR signaling via an association with $p56^{kk}$ activity (48).

We demonstrated that CD28 costimulation induces a DP phenotype in the human $CD8^+$ subset (Fig. 5). This agrees with findings by others, who demonstrated that a DP phenotype can be induced in human $CD8^+$ lymphocytes derived from peripheral blood, umbilical cord blood, fetal spleen, or fetal thymocytes (17, 18). In contrast, stimulation with anti-CD3 or anti-CD28 mAb alone failed to promote CD4 expression in the $CD8^+$ subset (18). While Blue *et al.* have demonstrated that Con A can induce a DP phenotype in a purified human $CD4^+$ subset (19), we did not observe similar effects in CD3/CD28-stimulated $CD4^+$ T-cells. The expression of the CD4 receptor on $CD8^+$ T-cells renders this population susceptible to HIV-1 infection (17, 18). Moreover, infectivity is enhanced by CXCR4 or CCR5 coexpression during CD28 costimulation in the same cell population (18).

While the role of CD4 expression on $CD8^+$ T-cells is unknown, human PBL routinely express a small percentage of DP cells (19–21). Occasional human subjects have been reported in which the percentage of DP lymphocytes expanded to 10–70% of PBL and was compatible with that associated with normal health (36, 37). Moreover, certain animal species such as rhesus macaques, cynomolgus monkeys, and swine express from 8 to 55% DP T-cells under normal health conditions (23–26, 41, 42). It is also interesting that, in these animals, the DP subset has been characterized as mature antigen-experienced cells which respond to recall antigen. Similarly, in humans it has been suggested that the DP subset exhibits a memory phenotype (41). While we are uncertain whether the DP subset in human blood has a $CD4^+/CD8^-$ or $CD8^+/CD4^-$ lineage, our *in vitro* data suggest that the $CD8^+$ subset is the more likely precursor, since $CD4^+$ T-cells do not express the CD8 receptor (Fig. 5). We have already mentioned that the acquisition of a CD4 receptor may be instrumental in long-term survival of the DP subset.

One of the hallmarks of immunosenescence is the expansion of memory T-cells at the expense of naive T-cells (49, 50). It is interesting, therefore, that the same animal species which exhibit an expanded DP lymphocyte pool also show a progressive rise in this subset during aging. For instance, Zuckermann and Husmann have shown that the percentage of DP lymphocytes in swine may increase from <2% in the first week of life to 30–55% by age 3 (41). Similarly, Nam *et al.* have described an increase in the DP subset from 1.3% at the age of 0.1 years to 8.6% in the third decade in cynomolgus monkeys (25). Our data also show a statistically significant increase in the number of DP T-cells in elderly humans compared to young people (Fig. 6). Interestingly, acquisition of $CD4^+$ by memory $CD8^+$ T-cells following multiple rounds of antigen-driven proliferation has also been observed in our studies on the process of replicative senescence in long-term culture (51).

From a diagnostic perspective, it is important to keep in mind that transient increases in the percentage of $CD4^+/CD8^+$ T-cells may occur during the course of infectious mononucleosis (39). A consistent or progressive increase in the percentage of DP cells has also been described in patients with lymphoblastic leukemia and lymphoblastic lymphoma (40), HTLV-1-mediated adult T-cell leukemia (52), Behcet's syndrome (53), and myasthenia gravis (54) and during expansion of large granular lymphocytes in the blood of humans (55). We propose that this increase represents an increase in the memory pool during aging and that staining for the DP subset may be a useful means of following that expansion.

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Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB

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The *ex vivo* priming and expansion of human cytotoxic T lymphocytes (CTLs) has potential for use in immunotherapy applications for cancer and infectious diseases. To overcome the difficulty in obtaining sufficient numbers of CTLs, we have developed artificial antigen-presenting cells (aAPCs) expressing ligands for the T-cell receptor (TCR) and the CD28 and 4-1BB co-stimulatory surface molecules. These aAPCs reproducibly activate and rapidly expand polyclonal or antigen-specific CD8+ T cells. The starting repertoire of CD8+ T cells was preserved during culture. Furthermore, apoptosis of cultured CD8+ T cells was diminished by this approach. This approach may have important therapeutic implications for adoptive immunotherapy.

Immunotherapy with CTLs holds promise for the treatment of cancer and infectious diseases[1,2]. Current studies of adoptive transfer in patients with human immunodeficiency virus (HIV), cytomegalovirus (CMV), and melanoma involve the infusion of T cells that have been stimulated, cloned, and expanded for many weeks *in vitro* on autologous dendritic cells (DCs), virally infected B cells, and/or allogeneic feeder cells[3–7]. These methods of cloning and expanding T cells have some drawbacks. Current cell culture techniques require several months to produce sufficient numbers of cells from a single clone[3,8]; this requirement may be a limiting factor in the setting of malignancy. Indeed, it is possible that the T cells that are finally infused into patients have a limited replicative capacity and therefore could not stably engraft to provide long-term protection from disease.

We have previously shown that magnetic beads coated with anti-CD3 and anti-CD28 antibodies can be used as aAPCs to support the long-term growth of CD4+ T cells[9]. However, we and others have found that beads or plates coated with anti-CD3 and anti-CD28 antibodies do not support the long-term growth of purified CD8+ T cells[10,11]. This limitation cannot be overcome by the addition of interleukin 2 (IL-2) to the culture medium[12]. We hypothesized that this limitation reflects a requirement for additional co-stimulation.

The tumor necrosis factor (TNF) receptor family member 4-1BB (CD137) was initially identified in receptor screens of activated lymphocytes[13]. The 4-1BB ligand is expressed by activated B cells, DCs, and monocytes/macrophages, all of which can act as APCs (ref. 14). Previous studies have shown that 4-1BB is a co-stimulatory molecule in the activation of T cells, and its signaling is independent from, albeit weaker than, CD28 signaling[13,15–17]. 4-1BB stimulation preferentially activates CD8+ T cells *in vitro* and amplifies generation of CTL responses *in vivo*[18]. The mechanism for this effect may involve improved survival of activated CTLs (ref. 19). Consistent with these data, co-stimulation of 4-1BB has been shown to have antiviral and antitumor effects[20–24]. These data led us to investigate 4-1BB as a candidate molecule that could promote *ex vivo* long-term growth of CD8+ T cells.

We sought to engineer a cell-based universal artificial APC system specifically optimized for the rapid expansion of human CTLs. The K562 erythromyeloid cell line was used as a scaffold, and we engineered the cells to stably express the human low-affinity Fcγ receptor, CD32, and the co-stimulatory molecule human (h) 4-1BB ligand. K562-CD32-4-1BB ligand cells coated with anti-CD3 and anti-CD28 antibodies can be used as aAPCs to stimulate the long-term growth of functional polyclonal and antigen-specific human CTLs. The addition of 4-1BBL was shown to maintain TCR diversity in T-cell cultures and to significantly reduce apoptosis of CD8+ T cells.

Results

Construction of aAPCs. To test the hypothesis that CD8+ T cells have distinct co-stimulation requirements for long-term growth, we designed a cell-based aAPC that could be genetically manipulated to express different co-stimulatory molecules in addition to CD28. We chose K562 cells because they do not express HLA proteins that would promote allogeneic responses, but they do express the T-cell interaction molecules ICAM (CD54) and LFA-3 (CD58) (Fig. 1A). Also, the eventual introduction of irradiated K562 cells into the clinical setting can be expedited because these cells are easily killed by

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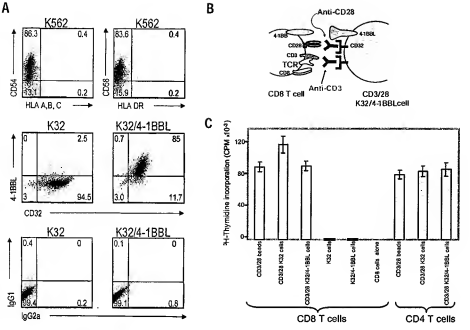

Figure 1. Construction of artificial APCs (aAPCs) from the parental K562 cell line. (A) Two-color flow-cytometric analysis of MHC I and II expression and CD54 and CD58 expression in parental K562 cells (top panels). Expression of CD32 and 4-1BBL in K32 (left) and K32/4-1BBL (right) cell lines is shown (middle panels). Isotype controls for the anti-CD32 antibody (IgG2a) and anti-41BBL antibody (IgG1) are shown for each aAPC (bottom panels). (B) Cartoon of engineered K32/4-1BBL aAPC interacting with a CD8+ T cell. K562 cells were transfected with the human Fcγ receptor CD32 ("K32 cells") to permit loading with anti-CD3 and anti-CD28 antibodies, and transfected with human 4-1BBL ("K32/4-1BBL cells") for added co-stimulation. (C) Proliferation of polyclonal CD4+ and CD8+ T cells stimulated with the indicated aAPCs was measured by [³H]thymidine incorporation between days 3 and 4 culture. T cells were stimulated with aAPCs as indicated, in the absence of cytokines. At 72 h, the cells were pulsed with [³H]thymidine and incubated for an additional 18 h before harvesting. Counts per minute values are shown as mean ± s.e.m. from triplicate cultures.

natural killer (NK) cells and are propagated in serum-free medium. We transfected and then cloned K562 cells expressing the human Fcγ receptor CD32 (K32 cells) to permit exogenous loading of anti-CD3 and anti-CD28 antibodies (Fig. 1A). Similarly, we generated the K32/4-1BBL line (Fig. 1A, B) by transfecting K32 cells with human 4-1BB ligand. Cultures were initiated by adding γ-irradiated aAPCs to fresh human CD8+ T cells prepared by negative selection (see Experimental Protocol).

K32 and K32/4-1BBL aAPCs efficiently activate human polyclonal CD8+ T cells. The aAPCs were tested for their ability to stimulate the initial activation and proliferation of primary CD8+ T cells. The T cells were stimulated with three different preparations of aAPCs: CD3/28 beads, K32 cells coated with anti-CD3 and anti-CD28 (K32/CD3/28), or K32/4-1BBL cells coated with the same antibodies (K32/4-1BBL/CD3/28). We found that the initial rate of growth of the T cells stimulated with all three aAPCs was equivalent, as judged by [³H]thymidine incorporation (Fig. 1C). We confirmed this observation by labeling fresh T cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) and tracking cell division during the first five days of culture (data not shown). We found that the K562 cell-based system was equivalent to CD3/28 beads for the induction of proliferation and cell division of CD4+ T cells (Fig. 1C and data not shown). No proliferation was seen in K562-based aAPCs, CD8+ T cells, or CD4+ T cells incubated separately (Fig. 1C and data not shown). Thus, the requirements for the initial rounds of CD8+ T-cell proliferation were satisfied equally by CD3/CD28 stimulation provided in the context of polystyrene beads or cell-based aAPCs, and the addition of 4-1BBL co-stimulation did not appear to have further benefit.

K32/4-1BBL aAPCs permit long-term expansion of human polyclonal CD8+ T cells. Next, we determined whether the aAPCs were sufficient to maintain long-term propagation of CD8+ T cells (Fig. 2A). CD8+ T cells were stimulated with aAPCs, but no exogenous cytokines were added to the medium. CD3/28 bead-stimulated cells failed to proliferate after the second stimulation with aAPCs, in agreement with previous studies[10,11]. Similarly, CD8+ T cells stimulated with CD3/28 in the context of K32 cells entered into a plateau phase of the growth curve within two weeks of culture, and no additional net growth of cells occurred after re-stimulation. In contrast, when CD8+ T-cell cultures were stimulated with K32/4-1BBL/CD3/28 aAPCs, they remained in exponential growth even after a third stimulation. This augmentation of long-term proliferation was reproducible, as the average increase in the total number of T cells was 410-fold higher in cultures stimulated with K32/4-1BBL/CD3/28 than in cultures stimulated with CD3/28 beads in six independent experiments.

Phenotypic analysis of cultures showed a progressive enrichment for CD3+CD8+ T cells after stimulation with K32/4-1BBL/CD3/28 aAPCs (Fig. 2B). The cell-based aAPCs rapidly disappeared from the cell cul-

ture, as evidenced by an inability to detect the irradiated K32/4-1BBL cells by flow cytometry after seven days (Fig. 2C); this finding was confirmed in large-scale experiments and also by RT-PCR for CD32 (data not shown). Thus, the mixed T-cell and aAPC culture yields a population of essentially pure T cells within one week.

Efficient propagation of antigen-specific cytotoxic T cells by K32/4-1BBL aAPCs. Immunotherapy with CD8+ T cells will likely require cells with antigen-specific cytolytic functions. To determine whether the K32/4-1BBL aAPCs could be used to expand antigen-specific CTLs, we used them to culture a population of MHC tetramer-sorted primary CD8+ T cells for 10 weeks (Fig. 3A). Purified CD8+ T cells obtained from an HLA-A*0201 donor were stained and sorted with an A*0201 MHC tetramer loaded with a flu matrix protein peptide (flu MP tetramer). The tetramer+ population was present at an initial frequency of 0.081% (Fig. 3B), which presumably was composed mainly of memory T cells. Cultures of tetramer- CD8+ T cells served as an internal control population of T cells to assess the growth potential and specificity of the tetramer+ population of CD8+ T cells. After bulk sorting, 16,000 cells each of CD8+fluMP+tetramer+ and tetramer- phenotype were stimulated with irradiated K32/4-1BBL/CD3/28 aAPCs (Fig. 3C). All cells were re-stimulated with K32/4-1BBL aAPCs at ~10-day intervals and recombinant human IL 2 (rhIL-2; 20 IU/ml) was added to the culture during the fourth week. No specific flu stimulation was provided during culture. Exponential growth curves of both populations of cells were obtained for several months. The 16,000 antigen-specific T cells yielded 1.5×10^9 cells after one month of culture, a number of cells sufficient for immunotherapy[2]. The substantial proliferative

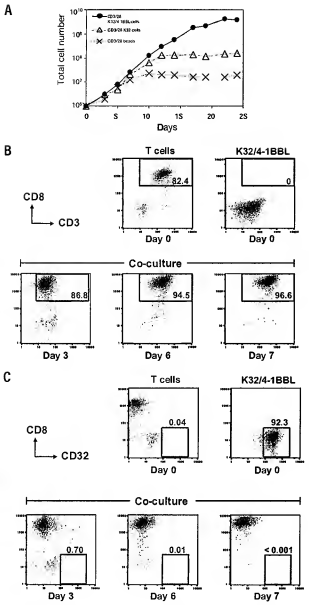

Figure 2. Long-term growth of primary polyclonal human CD8⁺ T cells stimulated with aAPCs in the absence of exogenous cytokines. (A) CD8⁺ T cells were stimulated with CD3/28 beads (X), irradiated K32 cells loaded with CD3/28 antibodies (Δ), or with irradiated K32/4-1BBL cells loaded with CD3/28 antibodies (•). T cells were stimulated with aAPCs on days 0, 10, and 20 of culture. (B, C) The purity of T cells and the fate of irradiated K32/4-1BBL stimulator cells were assessed by staining for CD3, CD8 (B), and CD32 (C) expression during the first seven days of culture. Variable numbers of red blood cells and platelets were contained in the input cultures; gating on cell size/debris was not used in this experiment so as to represent all cells in the culture. Viable cells are indicated by gating on propidium iodide to exclude dead cells. Results are representative of >10 different experiments, each with a different donor.

capacity of the CD8⁺ T cells that remains after 30 days of culture suggests that these CTLs could have substantial long-term engraftment potential after adoptive transfer.

To determine if antigen specificity of the expanded populations was maintained during culture, cells were stained with flu MP tetramer (Fig. 3B). On day 17, the population that was initially sorted as flu MP tetramer⁺ was 61.7% CD8⁺flu MP tetramer⁺, while the population that was sorted as flu MP tetramer⁻ had negligible staining. The percentage of tetramer⁺ cells in culture declined somewhat over time, but remained at >20% through day 60 (data not shown). Similar results were obtained with T cells from another HLA A*0201 donor, in which on day 26 of culture, the population sorted as flu MP tetramer⁺ was 49% CD8⁺ flu MP tetramer⁺ and again remained at >20% through day 60 (data not shown). Thus, a single round of selection for CD8⁺ cells with the desired specificity is sufficient to maintain acceptable purity of CD8⁺ cells cultured on K32/4-1BBL/CD3/28 aAPCs.

To examine the effector function of the cultured T cells, the antigen-specific cytolytic activity of the flu MP tetramer⁺ and tetramer⁻ cultures was determined by ^{51}Cr-release assays on days 26, 30, and 56 of *ex vivo* expansion (Fig. 3D and data not shown). The HLA-A*0201 TAP-deficient T2 cell line, pulsed or unpulsed with the flu MP peptide, was used as a target population. At all time points, flu MP tetramer⁺ cells displayed potent cytotoxicity for flu-MP peptide-pulsed targets. Flu MP tetramer⁺ cells did not kill unpulsed targets, and the flu MP tetramer⁻ cells did not kill either pulsed or unpulsed target cells. Neither effector population killed the parental K562 cells, suggesting that killing was MHC-restricted and not directed at K562 alloantigens (data not shown). Similar results were obtained with both donors (data not shown).

Maintenance of diverse TCR repertoire by K32/4-1BBL aAPCs. Given the finding that many tumor antigens are self-antigens, adoptive immunotherapy will require the isolation and propagation of T cells with generally low-affinity TCRs. Therefore, it is desirable that the culture system propagate T cells with uniform efficiency. To compare the properties of the cultures grown with aAPCs, cultures of CD8⁺ T cells grown on CD3/28-coated beads, K32/CD3/28, and K32/4-1BBL/CD3/28 aAPCs were assessed for maintenance of the initial TCR repertoire. CDR3 size length analysis of TCR β-chains was used because it permits sensitive detection of clonal T-cell outgrowth. We have previously shown that CD3/28-coated beads can maintain diverse CD4⁺ T-cell populations for several months in culture[9]; however, dramatic perturbations of the input CD8 repertoire occurred after two weeks of culture on these beads (data not shown). In contrast, CD8⁺ T cells cultured on K32/4-1BBL/CD3/28 aAPCs maintained CDR3 size length distributions that were similar to the input population of T cells (Fig. 4). The addition of 4-1BBL appeared to account for the preservation of the repertoire, because cultures of CD8⁺ T cells on K32/CD3/28 aAPCs did not maintain a comparably diverse repertoire (Fig. 4).

K32/4-1BBL aAPC stimulation enhances survival of human CD8⁺ T cells upon re-stimulation. Because the initial growth rate of CD8⁺ T cells stimulated with three different aAPCs was similar, we hypothesized that the increased overall growth observed in K32/4-1BBL/CD3/28 stimulated T cells was due to improved survival. We therefore determined the relative effects of the various aAPCs on expression of *Bcl-xL* and *IL-2*, two genes involved in T-cell survival and proliferation, respectively. Quantitative real-time RT-PCR was used to determine the levels of steady-state mRNA coding for Bcl-xL and IL-2 (Fig. 5). In all cultures, Bcl-xL and IL-2 expression was upregulated compared with resting cells one and three days after the first stimulation, and by day 10, *Bcl-xL* and *IL-2* gene expression had returned to resting levels. However, one to three days after re-stimulation, only CD8⁺ T-cell cultures that were stimulated with the K32/4-1BBL/CD3/28 aAPCs had increased levels of Bcl-xL and IL-2 mRNA. In contrast, CD8⁺ T cells that were stimulated with beads or K32/CD3/28 cells did not re-induce Bcl-xL or IL-2 expression after a second stimulation (Fig. 5A and B, respectively). Together these data suggest that 4-1BB co-stimulation provides a survival signal that is critical for subsequent but not the initial stimulation of CD8⁺ T-cell proliferation.

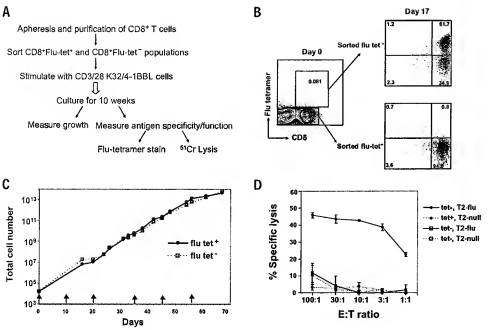

Figure 3. Propagation of antigen-specific cytotoxic T cells from an HLA A*0201 donor using K32/4-1BBL aAPCs. (A) Schematic of the experimental protocol.(B) Specificity of cell cultures as assessed by MHC tetramer staining. CD8+ T cells were stained with anti-CD8 antibody (x-axis) and A*0201 tetrameric MHC (y-axis) loaded with influenza matrix protein peptide (fluMP). Left panel: Initial cell population of T cells on day 0, with gates showing the cells sorted into CD8+flu-tet+ and CD8+flu-tet- populations. Right panels: tetramer staining of CD8+flu-tet+ (top) or CD8+flu-tet- (bottom) cultures after expansion on K32/4-1BBL cells for 26 days. (C) Growth curve of the sorted CD8+ T-cell populations. T cells were sorted into CD8+ fluMP tetramer+ (•) or CD8+ fluMP tetramer− (□). The sorted T-cell populations were then stimulated with irradiated K32/4-1BBL cells loaded with CD3/28 antibodies as indicated (arrows). rIL-2 was added to the cultures beginning on day 28. The total cell numbers are depicted in a semi-log plot of cell number vs. days in culture. (D) Cytotoxicity of flu-specific T cells after expansion on K32/4-1BBL aAPCs for 26 days. ^{51}Cr-release assays were done using TAP-deficient HLA A*0201 T2 target cells pulsed (circles) or unpulsed (squares) with the fluMP peptide. Antigen-specific cytotoxicity was also examined by comparing CD8+ fluMP tetramer+ cells (closed symbols) to CD8+FluMP tetramer− cells (open symbols). Values shown as mean ± s.e.m. of triplicate cultures. Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios. The entire protocol is representative of three experiments, each from different donors.

Compared with microspheric aAPCs, cell-based aAPCs may permit better formation of the immunological synapse as a result of the fluidity of the APC membrane[36]. Furthermore, the present system employing K562 cells as the scaffold has several other advantages for use in the clinic: they lack MHC expression, are mycoplasma-free, and have been adapted for growth in serum-free medium. In addition, these aAPCs can be used "off the shelf" to expand populations of CD8+ T cells from any donor in a short time period; this is particularly advantageous in the setting of progressive malignancy. Finally, *ex vivo* activation of CTLs for adoptive immunotherapy circumvents the numerous defects in the function of DCs that have been observed in cancer patients[37].

The K562 cell-based aAPC system is able to maintain exponential growth of CD8 memory cells for at least two months *in vitro*. Based on a starting cell population of 10^4 influenza-specific CD8+ T cells, we obtained a sufficient number of CTLs for therapy after only 30 days of culture. Because the starting number of antigen-specific CD8+ T cells could be isolated from only 100 ml of blood, given an initial frequency of 0.05%, it would be possible to decrease the culture time to only two weeks by doing a lymphopheresis and isolating 10^5–10^6 antigen-specific CD8+ T cells. High-speed cell sorting or magnetic bead separation can isolate sufficient CD8 memory cells for initial culture on K32/4-1BBL aAPCs coated with anti-CD3 and CD28 antibodies. Alternatively, it is possible to decorate the K32/4-1BBL aAPCs with the desired

We next assessed the viability of the CD8+ T cells stimulated by the various aAPCs during culture by fluorescent staining with annexin V and propidium iodide (Fig. 6). In the bead-stimulated cultures, viability gradually decreased in the first 10 days, and then dropped precipitously as only 6% of cells were viable on day 20. In the T-cell cultures stimulated with K32 aAPCs, T-cell viability seven days after the second stimulation was improved compared with bead-stimulated cells; however, most of the cells died by day 20. In contrast, K32/4-1BBL/CD3/28-stimulated CD8+ T-cell cultures were >70% viable throughout culture. Together these results show that the addition of 4-1BBL co-stimulation prevents apoptosis and preserves the starting repertoire of CD8+ T cells.

Discussion

We have developed aAPCs expressing 4-1BB ligand and decorated with anti-CD3 and anti-CD28 antibodies that can promote the rapid and long-term growth of purified polyclonal and antigen-specific CD8+ T cells. The K562 cell-based aAPCs have several advantages over previous culture systems that employ aAPCs constructed from beads or cells[38].

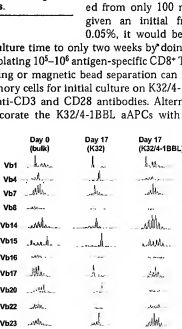

Figure 4. Maintenance of the TCR Vβ repertoire in polyclonal CD8+ T cells after expansion with K32/4-1BBL aAPCs. T cells cultured on K32 or K32/4-1BBL aAPCs from the growth curve shown in Figure 2 were assessed for the CDR3 length distribution. The indicated TCR Vβ family is shown at baseline, and after 17 days of culture.

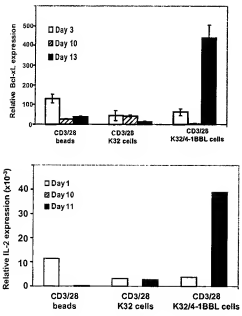

Figure 5. Expression of genes involved in T-cell growth and survival after stimulation with aAPCs. Real-time quantitative RT-PCR of Bcl-xL (mean ± s.e.m.) (A) or IL-2 (B) mRNA in polyclonal CD8⁺ T cultures. Y-axis, -fold expression of Bcl-xL or IL-2 relative to day 0 of culture. All cultures were stimulated with aAPCs on days 0 and 10. Results are representative of three different experiments with different donors.

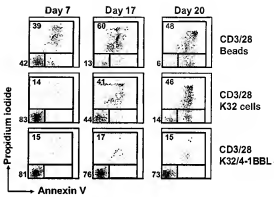

Figure 6. Distinct effects on apoptosis in cultures of polyclonal human CD8⁺ T cells stimulated with various aAPCs. Flow-cytometric analysis of cultured cells stained with FITC-labeled annexin V (x-axis) and propidium iodide (y-axis). The three rows represent different aAPCs used for stimulation. The columns represent days in culture. All cultures were stimulated with aAPCs on days 0 and 10. Data shown are not gated. Results are representative of three experiments with different donors.

tetramer so as to culture antigen-specific T cells *de novo*, and obviate the need for a separate cell isolation procedure.

One implication of this culture system is that the CTLs retain a substantial replicative capacity after culture with the K32/4-1BBL/CD3/28 aAPCs, even after reaching therapeutic numbers for clinical infusion. While previous investigators have noted long-term qualitative persistence of CTLs in human adoptive transfer protocols, the quantitative level of sustained engraftment has been low[28-31]. Therefore, our results may have therapeutic implications because sustained high-level engraftment of human CTLs has not been achieved to date.

Several mechanisms appear to account for the improved growth and repertoire of K32/4-1BBL/CD3/28-stimulated CD8⁺ T cells. We found markedly improved survival of CD8 cells after repeated stimulation with K32/4-1BBL/CD3/28 aAPCs compared with CD3/28-coated beads. We and others have observed that CD8⁺ T cells stimulated with anti-CD3/CD28 initially produce IL-2 (ref. 12), but unlike CD4⁺ cells, become unresponsive when re-stimulated *in vitro* with anti-CD3 and anti-CD28. However, with the addition of 4-1BB co-stimulation, CD8⁺ T cells have increased expression of IL-2 and Bcl-xL, improved survival, and continued proliferation after re-stimulation with anti-CD3/CD28. Thus, 4-1BB stimulation in this context overcomes the previously described activation-induced nonresponsiveness[19]. Because exogenous IL-2 does not overcome activation-induced nonresponsiveness in human CD8⁺ cells, it follows that the independent expression of other genes, such as Bcl-xL, is essential for the survival effect of 4-1BB signaling. However, other studies have shown that Bcl-xL induction alone, without complete IL-2 receptor signaling, is not sufficient to mediate cell survival or growth[32,33].

Not all clinically useful antigens are presently characterized as MHC-restricted epitopes, and the library of MHC tetramers for many HLA types remains limiting. Therefore, we have also used K32/4-1BBL/CD3/28 aAPCs to expand CTLs that have been previously enriched for a particular antigen specificity by priming with autologous DCs that have been pulsed with apoptotic bodies of autologous tumor (unpublished data). Thus, K32/4-1BBL/CD3/28 aAPCs are likely to be complementary to many methods, including MHC tetramer sorting[34,35] or priming with autologous DCs or other artificial APCs (ref. 25) that enrich for antigen-specific CTL populations. Thus far, we have only tested the K32/4-1BBL/CD3/28 aAPCs for their ability to expand memory or primed T cells; it will be desirable to expand naive CD8 cells as a source of the "self" repertoire for tumor immunotherapy[36-38]. Expanding low-avidity, self-reactive T cells[39] that can differentiate into memory cells[40] may serve as a useful approach to derive therapeutic numbers of self-reactive CTLs.

Given that clinical-grade preparations of anti-CD3 and CD28 antibodies are currently available, and that K32/4-1BBL aAPCs can be grown in serum-free medium, it is likely that this system has therapeutic potential for clinical adoptive immunotherapy for patients with cancer and viral diseases. Furthermore, these aAPCs may be useful for the *in vitro* propagation of CTLs for experimentation.

Experimental protocol

Cloning and construction of cell-based aAPCs. Human CD32 was cloned from neutrophils into the pcDNA 3.1 neo vector (Invitrogen, Carlsbad, CA) and transfected by electroporation into K562 cells obtained from the American Type Culture Collection (ATCC; Manassas, VA); K32 cells were cloned by fluorescence-activated cell sorting (FACS). Similarly, h4-1BB ligand was cloned from B cells into the pcDNA3.1 hygro vector (Invitrogen) and transfected into K32 cells before FACS.

CD8⁺ T-lymphocyte preparation and K562 cell culture. Fresh peripheral blood lymphocytes were obtained by leukopheresis and elutriation, and CD4⁺ T cells were purified by negative selection using the OKT8 antibody (ATCC) as described[41]; CD8⁺ T cells were purified identically but substituting the OKT4 antibody (ATCC) for the OKT8 antibody. All cultures were maintained in AIM V (GIBCO BRL, Life Technologies, Grand Island, NY) with 3% human AB serum (BioWhittaker, Walkersville, MD). Human IL-2 (Chiron Therapeutics, Emeryville, CA) was added at 20 IU/ml where indicated.

T-lymphocyte stimulation and long-term culture. At each time that lymphocytes were stimulated, the K562 cell-based aAPCs were irradiated with 100Gy (10,000 rads) and washed twice into T-cell culture medium. Cell-based aAPCs were then loaded with anti-CD3 (OKT3) and anti-CD28 monoclonal antibodies (mAbs; 9.3) at 0.5 μg/ml for 10 min at room temperature. Unwashed, antibody-loaded aAPCs were then mixed with CD8⁺ T cells at a

1:2 ratio of K562 to T cells. The T-cell concentration was maintained at 0.5×10^6 cells/ml throughout culture, and up to 100×10^6 T cells were maintained in flasks. Anti-CD3/28 bead stimulation was performed as described[9]. Cultured T cells were monitored for cell volume and enumerated on a Coulter Multisizer II (Miami, FL) every 2–3 days, and re-stimulated at 7- to 10-day intervals when the mean lymphocyte volume reached 200–250 fl.

Flow cytometry and FACS sorting. Cells were stained with antibodies (and/or MHC tetramers) at 4°C, and analyzed on a FACSCalibur (BD BioSciences, Mountain View, CA). Apoptosis assays were conducted according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Cell sorting was performed on a MoFlo cell sorter (Cytomation, Fort Collins, CO). All flow cytometry data were analyzed with FlowJo software (TreeStar, San Carlos, CA).

Real-time PCR and TCR Vβ repertoire analysis. Real-time PCR was done and normalized to 28S ribosomal RNA (rRNA) levels as described[42]. The diversity of TCR Vβ repertoire was assessed by determination of CDR3 size lengths by multiplex PCR as described[43].

^{51}Cr-release assays. Target T2 cells (ATCC) were pulsed with 10 μM flu peptide (GILGFVFTL) or left unpulsed before labeling with chromium-51 (PerkinElmer Life Sciences, Inc., Boston, MA). After a 4 h incubation of effectors with targets, radioactivity was counted from an aliquot of supernatant. Specific lysis was calculated by standard methods.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology *web site (http://biotech.nature.com) for details.*

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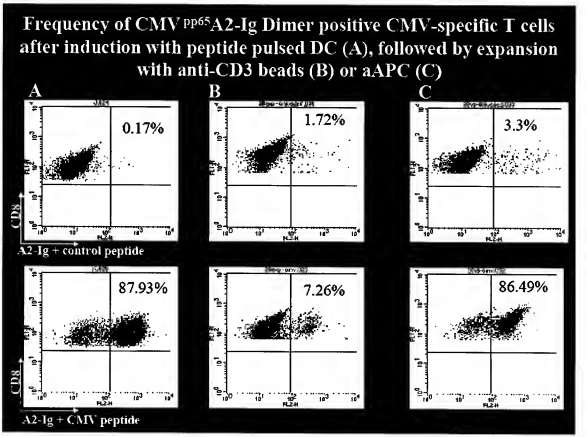
Frequency of CMV pp65 A2-Ig Dimer positive CMV-specific T cells after induction with peptide pulsed DC (A), followed by expansion with anti-CD3 beads (B) or aAPC (C)
A
B
C
0.17%
1.72%
3.3%
CD8
A2-Ig + control peptide
87.93%
7.26%
86.49%
CD8
A2-Ig + CMV peptide